UNIVERSITY OF ALBERTA

FAC/MS – A NOVEL HCV NS3 SCREENING ASSAY

By

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©

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List of Abbreviations

ACN	acetonitrile
AD	activation domain
AH	amphipathic helix
ALT	alanine aminotransferase
BD	binding domain
BSA	bovine serum albumin
Bt	column capacity
BVDV	bovine diarrhoeal virus
C	core
CD	cluster differentiation
Cha	cyclohexyl alanine
CDR	complementarity-determining region
cDNA	complimentary DNA
DNA	deoxyribose nucleic acid
DHBV	duck Hepatitis B virus
DTT	dithiothreitol
E	envelope

E.coli	Escheria coli
EIC	extracted ion chromatograph
EIF	eukaryotic initiation factor
ER	endoplasmic reticulum
ESI-MS	electrospray inonizer mass spectrometer
FACS	fluorescent activated cell sorter
FAC/MS	frontal affinity chromatography coupled on-line to a mass Spectrometer
FPLC	fast protein liquid chromatography
fPR	D-phenylalanine-proline-arginine tripeptide
Gp	glycoprotein
GlcNAc	N-acetyl glucosamine
Glcp	glucose pyranoside
GlcpNAc	N-acetyl glucosamine pyranoside
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCC	hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPLC	high pressure liquid chromatography
HVAP-A	human vesicle-associated membrane protein-associated protein A
HVR	hyper variable region
IC	inhibitory concentration

IFN	interferon
IRES	internal ribosome entry site
ISDR	interferon sensitivity determining region
KDa	kilodalton
LC	long chain
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
MAb	monoclonal antibody
Man	mannose
Manp	mannose pyranoside
MS	mass spectrometry
NANBH	Non-A, non-B Hepatitis virus
NHS	N-hyrxoysuccinimide ester
NS	nonstructural
OAc	acetate
OGMGG	β-D-GlcpNAc-(1→2)-[β-D-GlcpNAc-(1→6)]-α-D-Manp(1→6)-β-D-Glcp-*O*-octyl glucoside
ORF	open reading frame
PCR	polymerase chain reaction
PEEK	Polyetheretherketone
PKR	double-stranded RNA-activated protein kinase
RdRp	RNA dependent RNA polymerase
RNA	ribose nucleic acid

RT	reverse transcriptase
SA-HRP	streptavidin conjugated to horse radish peroxidase
SCICLOPPS	an acronym for the split-intein method to produce cyclic peptides
SELEX	systematic evolution of ligands by exponential enrichment
SPA	scintillation proximity assay
Tert	tertiary
Trx	thioredoxin
UTR	untranslated region
Y2H	yeast two hybrid

Chapter 1

Introduction

I. *Epidemiology, Discovery and Therapy of HCV*

Epidemiology

Hepatitis C virus (HCV) is the leading cause of liver disease worldwide, as well as the primary cause of liver transplantation in North America and Europe. [2] It is estimated that approximately 3% of the world's population is infected with HCV, however most of the chronic carriers are assumed to be undiagnosed. [2] The seroprevalence rate is about 1% in Western Europe and North America, 3-4% in some Mediterranean and Asian countries and as high as 10-20% in parts of Central Africa and Egypt. [191] The magnitude of HCV infection was first appreciated in the 1970s, when specific diagnostics were developed for the hepatitis A virus (HAV) and the hepatitis B virus (HBV). These viruses were responsible for liver associated diseases transmitted via enteric and parenteral transmission respectively. [27,35] It soon became clear that many cases of hepatitis were not caused by HAV or HBV. These were referred to as non-A, non-B hepatitis (NANBH) until the identification of one causative agent, HCV in 1989. [27]

HCV is transmitted primarily by parenteral exposure to blood from an infected individual. Over the last few decades, most of those infected with HCV likely acquired the disease from poorly sterilized needles and instruments used by intravenous drug users, medical and dental practitioners, and tattooing and body piercing parlours. [35] In fact, the highest known prevalence in a given population is in Egypt (24%), which is likely the result of an extensive campaign to combat schistosomiasis with parenteral drug administration with reusable needles. [35] Until diagnostic tests for HCV were made available in 1990, unscreened blood transfusions were another major source of HCV. Fortunately, the routine screening of donor blood has virtually eliminated the spread of HCV via blood transfusions. Today, in the industrialized countries, the single most important mode of transmission of HCV is the use of intravenous illegal drugs. [133] Occupational needle stick injuries from HCV-positive sources result in seroconversion in about 3% of recipients. [36] Infection from sexual contact appears to be uncommon as partners of HCV-positive hemophiliacs have very low rates of infection (2.7-2.9%), and most of the few positive partners had alternative risk factors. [20,72] Vertical transmission has been observed, but the risk estimates are less than 5%, unless the mother is co-infected with HIV. [133]

The course of an HCV infection is not predicatable, as the severity of the infection can vary from carrier to carrier. Some of the symptoms of an acute infection resemble those of the common flu, including fatigue, loss of appetite and other non-specific symptoms. However, the majority of those infected with

HCV (about 75%) do not experience the symptoms of an acute infection. [133] Approximately 70-80% of those exposed to HCV develop a chronic infection; in the majority disease is sub-clinical. Typically it is only after 20 years that the serious-to-life-threatening liver associated diseases emerge. In approximately 20% of chronically infected patients the disease progresses to liver cirrhosis, which is highly associated with hepatocellular carcinoma (HCC). Once cirrhosis is established, the rate of HCC development is 1-4% per year. [133] End stage liver disease can occur in cirrhotic patients and liver transplantation is often the only treatment option. With the limited availability of organs for transplantion, there is an enormous pressure to develop improved antiviral therapies for HCV.

Extrahepatic pathologies associated with chronic HCV infection include mixed cryoglobulinemia (in 1-2% of patients), glomerulonephritis and porphyria cutanea tarada. [204] Recently, chronic HCV has been associated with non-Hodgkin's lymphoma and type 2 diabetes mellitus, however the role of HCV in the etiology of these remains to be confirmed. [133]

Identification of HCV

With the development of more sensitive and powerful molecular biology techniques, beginning in the 1980s, it became easier to identify new infectious diseases. Using plasma from a NANBH-infected chimpanzee [17], Dr. Michael Houghton lead a team in the Chiron Corporation to identify the new virus – HCV.

They used a lambda phage cDNA library from the nucleic acids extracted from the serum of a chimpanzee "infected" with serum from a NANB patient. The serum of a patient with chronic NANBH was used as the antibody source to screen the library, and clone 5-1-1 expressed an antigen that was recognized by the patient's serum antibodies. [27] The origin of the clone was shown to be a 10000 kb single-stranded RNA molecule with one continuous open reading frame (ORF) now identified as HCV-specific. The 5-1-1 antigen became the first serological test for HCV. The team subsequently found that at least 80% of patients with post-transfusion NANBH possessed antibodies to 5-1-1. [27]

Treatment of HCV

The current antiviral regimens for HCV rely heavily on interferon alpha (IFN-α) and have undergone a significant improvement in the last decade. [44] The pegylated form of IFN-α together with ribavirin is now the "gold standard" for treatment and yields a sustained response in more than 40% of patients infected by genotype 1 viruses (the most prevalent form in North America), and roughly 80% of carriers with genotypes 2 and 3. [25] Despite these advances, treatment with pegylated IFN-α and ribavirin has several disadvantages. This combination therapy has significant side effects and is not easily tolerated by 20-30% of patients. [63] Side effects include leukpenia, thrombocytopenia, thyroid dysfunction, hair loss and mental changes including anxiety, irritability and depression. Furthermore this treatment is expensive (approximately $20,000 CDN for one year) and often beyond the financial resources of patients without insurance including drug benefits. The frequency of treatment failure, compounded by the high prevalence of this disease, points to the need for more specific, less costly and less toxic antiviral therapies for HCV. Analogously to the HIV antiviral research, attention has focused on developing agents that inhibit the key viral enzymes, specifically the serine protease NS3-4A, and the NS5B polymerase. [42]

II. Molecular Biology of HCV & Targets for Antivirals

The steps of the viral life cycle are summarized in Figure 1-1. HCV is a positive-stranded RNA virus of the Flaviviridae family, subgenera hepacivirus. The RNA is approximately 9.6 kb in length and is translated into a single polyprotein of about 3000 amino acid residues. [123]. The organization of the genome closely resembles that of other pestiviruses such as the bovine viral diarrhea virus (BVDV). Worldwide there are at least 6 major genotyopes for HCV, which in addition to differences in length, show diversities up to 30% in their nucleotide sequences. [166,167]

The first few steps of the cycle, binding to the cell, endocytosis, virion membrane fusion and uncoating are not well understood to date. Only recently has progress been made towards producing modified cell culture systems and a small animal model to study these phenomena.

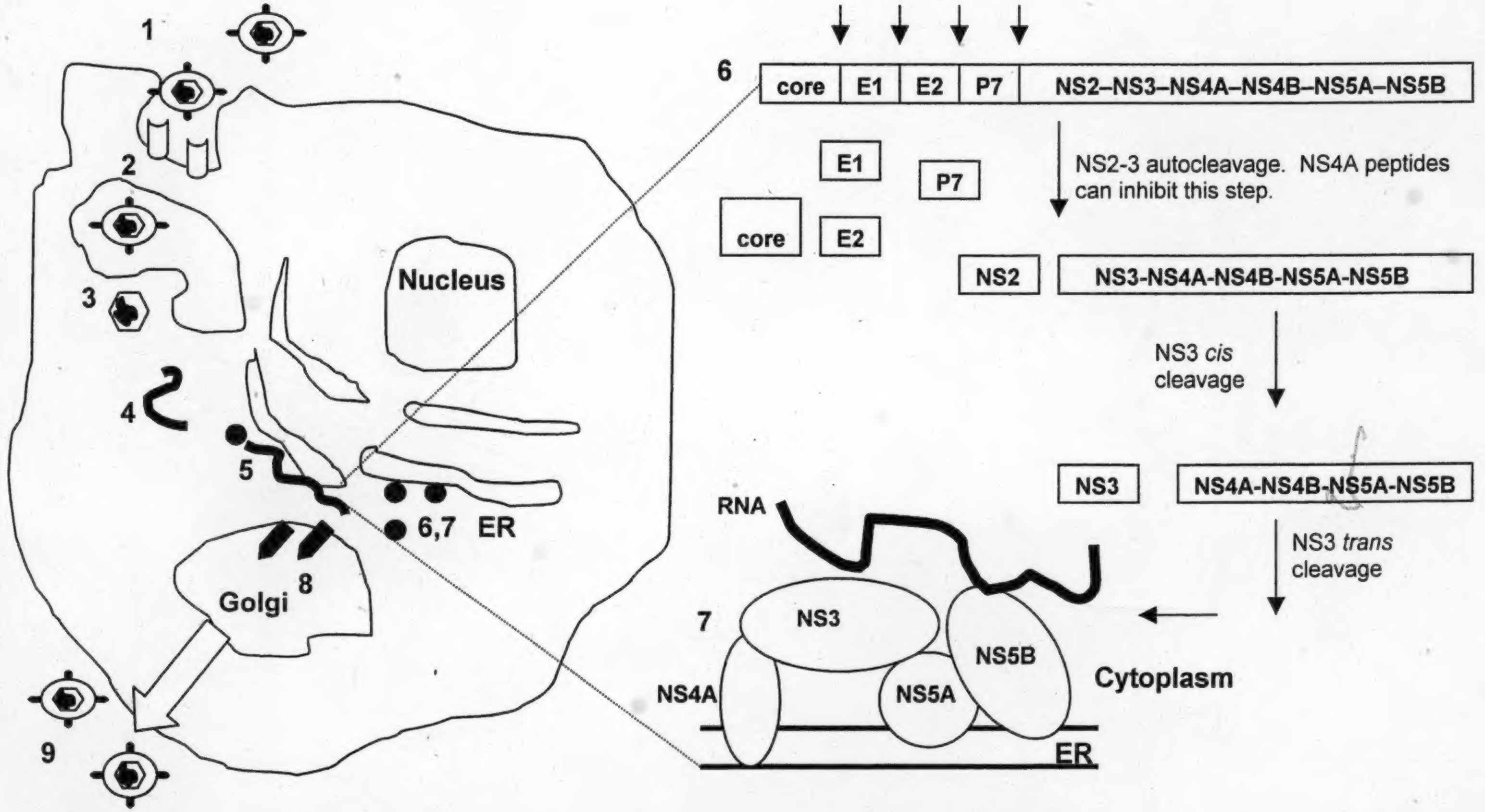
1
2
3
4
5
6,7
ER
8
9
Nucleus
Golgi
6
core
E1
E2
P7
NS2–NS3–NS4A–NS4B–NS5A–NS5B
NS2-3 autocleavage. NS4A peptides can inhibit this step.
core
E1
E2
P7
NS2
NS3-NS4A-NS4B-NS5A-NS5B
NS3 *cis* cleavage
NS3
NS4A-NS4B-NS5A-NS5B
NS3 *trans* cleavage
RNA
7
NS3
NS4A
NS5A
NS5B
Cytoplasm
ER

Fig. 1.1. Outline of HCV replication. (1) Binding of HCV virion to host receptor - CD81 and/or LDL-R. (2) Receptor mediated endocytosis. (3) Viral membrane fusion and release of core particle into cytoplasm. (4) Uncoating step. (5) Tranlation mediated by IRES and host translation apparatus. (6) Polyprotein production and processing. (7) Replication complex assembly at the surface of the ER. Positive and negative stranded RNA is produced by the viral RNA dependent RNA polymerase. (8) Virion assembly in a pre-Golgi compartment. (9) The HCV virionlikely buds from the golgi membrane . Adapted from a diagram by Dr. M. Joyce (Tyrrell Laboratory).

1. Attachment and Entry

HCV appears to be primarily a hepatotropic virus – based on the non-structural (NS) proteins and viral RNA which have been detected in the livers of both patients and experimentally infected chimpanzees. A liver-specific receptor, however, has not been unambiguously identified. [8] There are two primary candidiates, the low-density lipoprotein receptor (LDLR) and CD81. Insight into the mechanism by which HCV gains entry into host cells will be critical for an understanding of primary infection and how it might be prevented.

One early observation that implicated the LDLR was that the buoyant density of HCV in infectious serum was less than the buoyant density of the viral particle from non-infectious serum. [16,77] The buoyant density (<1.06 g/mL) suggested an association with the low-density lipoprotein (LDL) fraction, an association which was subsequently verified. [16,77] HCV isolated from patients whose serum is non-infectious are complexed with antibodies leading to a higher buoyant density. [77] The mechanism whereby the LDLs bind to the viral envelope is not understood but this binding may be an intermediary of the infectious process. [31] The use of the LDLR by HCV as a hepatocyte receptor for the virus is an attractive explanation for its hepatotropism. Furthermore the internalization of the LDLR by receptor-mediated endocytosis could explain the initial step of viral binding and entry. Evidence to corroborate this hypothesis came from experiments showing a direct correlation between the level of cell surface LDLR and the number of HCV-RNA positive cells determined by *in situ* hybridization. HCV does not bind to COS-7 cells unless they are transfected with the gene for the LDLR.[131] Perhaps the best evidence implicating the LDL-R to date is the observation that antibodies to the LDL-R were able to block viral entry *in vitro.* [1]

The second candidate, CD81, is a tetraspanin, which is expressed on a number of different cells, including B lymphocytes and liver cells. It was found to bind to HCV envelope protein E2 in cDNA library screening assays. [146] The binding of E2 to CD81 *in vitro* has been thoroughly studied by mutagenesis with much of the binding localized to a large extracellular loop of CD81. [75] It has also been shown that human CD81 alone is not sufficient to permit HCV entry *in vitro.* [125] However CD81 has been demonstrated to be a coreceptor for HCV using an E1/E2 pseudotyped retroviral particles[37], and expression of CD81 in previously unsusceptible liver cell lines subsequently can render them susceptible to HIV-HCV pseudotyped viruses. Small molecules that inhibit the interaction of the envelope proteins with CD81 have already been reported as a possible antiviral therapy for HCV.[188]

Some of the pathologies associated with HCV support the possibility of this receptor being employed by HCV. For instance, non-Hodgkin's lymphoma and mixed-type II cryoglobulinemia, both B-cell lymphoproliferative disorders, are observed in HCV infected patients. As CD81 is a co-signalling molecule on B-cells, it is interesting to speculate whether E2 binding may cause these syndromes. [39] Recent advances in cell culture models have also found B-cells capable of supporting HCV replication, which suggests that B-cells are at least a potential extra-hepatic resevoir for the virus *in vivo.* [172] This would suggest that if HCV is genuinely infecting B lymphocytes, another factor in addition to CD81 must be involved.

Although CD81 and the LDLR have been identified as leading candidates for the HCV receptor on cells, a variety of other possible receptors have been reported including: the asialoglycoprotein receptor [155], L-SIGN [68] and a scavenger receptor. [156] However, the significance of any of these receptors *in vivo* remains to be substantiated.

Although there are apparently no reports of fusion inhibitors in the literature, the Technology Transfer office of Tulane University has reported that researchers have isolated peptides that are capable of preventing HCV viral particle fusion. As such peptides have been developed to treat HIV infection, perhaps a similar therapy can be developed for HCV. However considerable basic research will be required before such peptides will find clinical use.

2. TRANSLATION, GENOME ORGANIZATION & REPLICATION

Following the entry and uncoating of the viral genome, the internal ribosome entry site (IRES) promotes the translation of the polyprotein, which is subsequently cleaved into the functional proteins. The structural proteins, envelope proteins 1 and 2 (E1,E2), p7 and Core (C), are either transmembrane penetrating (E1, E2 and p7) or are associated (C) with the endoplasmic reticulum (ER) membrane of the infected cell. It is believed the structural proteins form hetero oligomers that eventually promote viral budding. The non-structural (NS) proteins include: NS2, a poorly characterized protease; NS3, a serine protease and a helicase; NS4A, a cofactor for NS3; NS4B, a protein of unknown function; NS5A, another not well-studied protein; and NS5B, a polymerase. The NS proteins associate with themselves and specific host proteins on the cytoplasmic side of the ER to form the replication complexes. The replication complexes use the viral genome as a template for the synthesis of the negative-stranded replicative intermediates. The minus strand, or possibly a positive and negative strand duplex, serves as the template for the synthesis of new positive stranded genomic RNA which can be recycled for further rounds of replication, protein synthesis or be packaged into virions. The details of viral packaging are unclear and researchers await new cell culture models to study the molecular details of the life cycle of the virus. [42]

5' Untranslated Region (UTR)

The 341 nucleotide length of the 5' UTR of HCV is considered unusually long for a positive stranded RNA virus. [32] Computer modelling and biochemical analysis suggests the viral RNA folds into complex secondary and tertiary structures.[31] Sequence analyses of 39 different viral isolates have shown very high sequence conservation in the 5' UTR, implying it likely has an important function for the virus.[62,151] There is no evidence of a specialized cap structure to allow a ribosome to bind and scan along the RNA for the most proximal AUG start codon to initiate translation. Instead, the extensive secondary structure of the 5' UTR acts as an IRES, allowing translation to occur in a cap-independent manner. A specific interaction between the HCV IRES and the 40 S ribosomal subunit and eukaryotic initiation factor eIF3 has been demonstrated.[90] A functional requirement for two of the subunits of eIF2 (eIF2γ and eIF2Bγ) was also demonstrated.[101] However the complex structure of the IRES is somehow able to replace the role of several of the other typical translation initiation factors.[88] An induced conformational change in the 40 S subunit appears to lead to the alignment of the ribosomal subunit P site with the initiation codon by a mechanism that resembles the role of the Shine-Delgarno sequences in prokaryotic translation.[90] The unique nature of the IRES function presents an attractive target in terms of selectivity for antiviral agents. A class of phenazine-like molecules has been described as potential inhibitors of HCV IRES function in mammalian cells.[190] In addition, vitamin B-12 was also found to cause stalling of the IRES-directed translation, and is being examined as a possible IRES inhibitor.[117,175]

Core

HCV core is a basic, 20 kDa protein and forms the nucleocapsid surrounding the RNA genome in the virion. [76] The 5' end of the core coding sequence is part of the IRES, and may be involved in the switch between viral polyprotein synthesis and subsequent viral RNA replication. [164] It is now been reported that the first 20 amino acids of core have been shown to bind and inhibit HCV IRES-dependent translation in certain cell lines but may also inhibit cap-dependent translation in other cell lines. The significance of these observations is not yet known. [107]

It addition to these functional aspects, core has also been implicated in pathogenesis of HCV by acting as an immunosuppressive agent. One report suggested core interacts with the cytoplasmic tail of the lymphotoxin-β receptor, a member of the tumour necrosis factor family, which may explain its ability to suppress apoptosis in tissue culture. [126] Core was also shown to prevent the IFN-α induced nuclear import of STAT transcription factors, particularly STAT1, with a decrease in the MxA protein expression. [128] Furthermore core can be cleaved at amino acid position 179 by an intramembrane signal peptidase leading to its cytosolic release. Surprisingly the core localized to the mitochondria the targeting domain being mapped to a stretch of 10 hydrophobic amino acids from the new C-terminus. Considering the role of the mitochondria in lipid metabolism and apoptosis, it will be of interest to determine core's function at this subcellular compartment. [158]

Capsid formation has been considered a target for antivirals[205] and thus some effort has been made to study capsid assembly. Cell free translation assays have shown capsid assembly can occur in the absence of intact membranes and independent of the signal sequence (prior to cleavage at position 171) and was inhibited by cultured liver cell lysates. Such a system might allow for mechanistic dissection of the assembly process and help in the discovery of antiviral that target this protein-protein interaction.[94]

F protein

This is a recently discovered 17 kDa unstable protein that is a result of a ribosomal frameshift in the core coding sequence. [195] Very little is known about this protein at present. [189] During HCV infections, however, patients develop an immune response to this antigen.

Envelope proteins E1 and E2

Following their translation, there is rapid cleavage at the N-termini of E1 (gp31) and E2 (gp 70) polyprotein junctions, however the E2/p7 junction is incomplete leading to mixed populations of E2 and E2/p7. [109,130] The reason for this incomplete processing is not yet clear. Both proteins are type I transmembrane (TM) proteins with N-terminal ectodomains and C-terminal transmembrane anchors. Transient expression systems have shown E1 and E2 interact to form oligomers and two pathways of E/E2 folding and assembly have been proposed (Figure 1-2).

In the nonproductive pathway, there are intermolecular disulphide bonds leading to V8 protease sensitive aggregates[46], regardless if a high expression (eg. viral) or low expression vector is used.[28] This in turn suggests this is an intrinsic property of the envelope glycoproteins. One explanation is that secretory proteins that are retained in the ER in a misfolded state can activate the transcription of intraluminal chaperone proteins such as GRP78 (BiP) through a stress signal. E2 was found to bind tightly to and also induce the expression of GRP78. Overexpression of GRP78 has shown to decrease the sensitivity of cells to cytotoxic lymphocytes.[108]

In the productive pathway the E1 and E2 proteins are thought to form a non-covalent heterodimer with intramolecular disulphide bonds. Although not proven, these were inferred from immunoprecipitation studies with a conformational specific antibody.[43,46] More recent studies of the envelope protein derived from infectious pseudotyped virus were also recognized by conformational-dependent antibodies.[138] The glycoprotein conformation was also sensitive to low-pH treatment which would likely be necessary to initiate fusion.

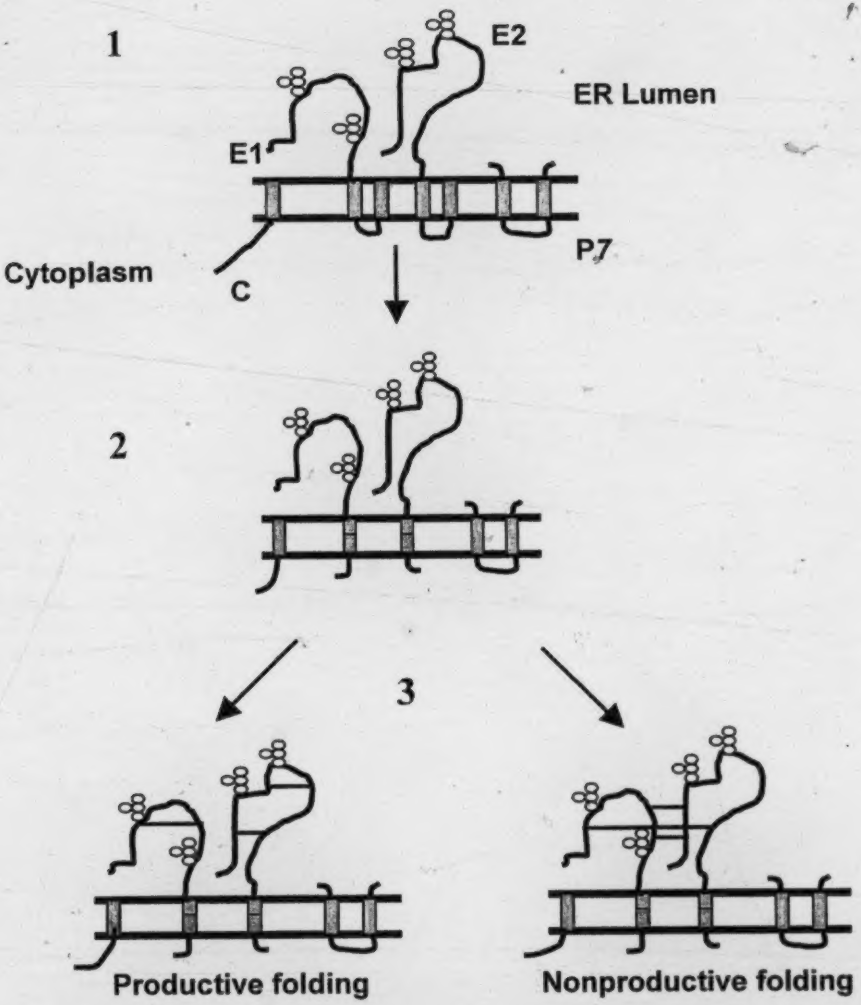

Fig. 1-2. Folding of envelope glycoproteins. (1) The polyprotein is cleaved by signal peptidases. The circles represent the high-mannose glycans. The dark transmembrane spanning boxes are the signal sequences. The envelope proteins remain in the ER and are not retrieved from the Golgi as suggested by glycan analysis and immunoloclaization studies. (2) Splicing event causing the envelope TM C-termini to contain part of the signal sequence. (3) Productive and non-productive folding pathways. In productive folding, E1 folding is assisted by E2. Intramolecular disulphide bonds occur leading to a noncovalent heterodimer. In nonproductive folding, intermolecular disulphide bonds occur between the two envelope proteins leading to aggregates. This retention suggests that budding might occur by invagination of the ER by way of the nucleocapsid and subsequently follow the secretory pathway. Transit through the Golgi would then modify the envelope glycoproteins associated with the particle leading to the more complex type glycans seen on virions recovered from the circulation of infected patients.

The interaction between the heterodimers is mediated by both the ectodomain and the TM regions. Pull-down assays with truncated forms in mammalian cells have shown the N-terminal sequences of E2 ectodomain (amino acids 415-500 of the polyprotein) are important, but deletion analysis of the ectodomain of E2 has yet to define a single region necessary for the non-covelant interaction. It should be noted that ectodomain interactions have been shown to be involved in oligomerization for other viral envelope proteins and is important for their fusogenic activity. The TM of the E2 is also important for dimerization as its deletion, replacement with the TM domain of another protein, or interruption by scanning alanine insertion mutagenesis prevents herterodimer interaction.

HCV E1 and E2 proteins appear to have an interesting form of intracellular trafficking. The envelope proteins are not targeted towards the Golgi but are actually retained in the ER.[48] Many ER resident protein are actually retrieved from the Golgi via C-terminal KDEL or KKXX sequences[137] but this is not the case in HCV. When expressed together the E1E2 heterodimer is located in an early compartment of the secretory pathway was revealed by immunolocalization studies.[43] Whether or not the proteins proceeded to the Golgi and were subsequently retrieved was answered by an additional immunolocalization study with nocodazole treatment.[48] Nocodazole is a drug that inhibits the polymerisation of free tubuline molecules by binding an arginine residue of the ß-tubulin subunit and thus prevents the intracellular trafficking between the Golgi and ER compartments. Treatment with and without the drug showed little difference in immunolocalization. Furthermore HPLC analysis of the glycans revealed only three species Man_9, Man_8 and $Man_7GlcNAC$. The lack of complex-type glycosylation excludes the possibility of their retrieval from the medial Golgi. Had the proteins entered the *cis*-Golgi the the glycans would have been processed to the Man5GlcNAC2 form because of exposure to Golgi α-mannosidase I.[48] The glycans should also be sensitive to Endo H treatment had they been processed in the Golgi but they were not, suggesting retention was most likely to have occurred. Lectin-binding studies with patient derived HCV particles have shown complex type glycans[153] which is probably occurring as the virus particle follows the exocytic pathway to the extracellular environment. This has not been possible to study yet because of the lack of cell culture models capable of generating such complete particles. Following this line of argument it has been shown that the HCV glycoproteins can bind L-SIGN (a liver -associated lectin for complex glycans) and internalize HCV particles into nonlysosomal compartments.[120] Such an interaction would not likely occur unless the HCV glycoproteins were at some point modified into a complex form from their initial high mannose form.

The first retention signal was demonstrated using a chimeric protein using the 29 amino acids of the TM of E2 and the ectodomain of CD4[34], which is normally expressed at the cell surface. A similar approach successfully demonstrated a second retention signal in the TM domain of E1.[33] Again, glycan analysis helped proved these are indeed static retention and not retrieval signal.

There is some evidence that the ectodomain of E1 (amino acids 290-333 of the polyprotein BK strain) also functions as a retention signal with CD4 chimeric proteins. Anti-E1 antibodies can detect some plasma membrane expression of other chimeric proteins meaning the signal might be strictly recognized. There are two highly conserved charged amino acid positions in the TM region between two hydrophobic patches of amino acids. In E1 there is a lysine and in E2 an aspartic acid and arginine. Mutagnesis of the lysine to an alanine in the E1 TM lead to cell surface expression of the CD4-E1 TM chimeric protein. Similarly, replacement of the aspartic acid and arginine residues with alanine lead to cell surface expression of E2.

The E2 protein, although highly immunogenic and a possible vaccine candidate, [55] is known to exhibit a very high degree of sequence variation in its N-terminal region, amino acids 1-27, which has been dubbed the hypervariable region (HVR1). The HVR1 is thought to be responsible for the generation of escape mutants to the host antibody response, as considerable variation in this region can be seen in quasi-species isolated from the same patient. [54] A second immune evasion function of E2 may be its ability to inhibit the activity of PKR, the double-stranded RNA-activated RNAase. The mechanism is thought to be attributable to a sequence in E2 that is similar to one that is autophosphorylated in PKR. The mechanism is likely more complex. [178,179]

The development of preventive vaccines for HCV is clearly of the utmost importance. Unfortunately, this has been hampered by the fact that there are six major genotypes and more than 30 subtypes worldwide, as well as the emergence of genetic variations (quasispecies) in infected individuals. [166] However, effective vaccine development for HCV remains a major goal of a number of research laboratories and biotechnology companies.

p7

It has been recently shown that HCV p7 is a polytopic membrane protein that crosses the membrane twice, with its N and C termini directed toward the extracellular environment. [24] Little is known about the function of p7 protein, however other pestiviruses posses an analogous of p7 for which some functional data is known. Deletion of the p7 gene from BVDV does not appear to affect RNA replication, but leads to the production of non-infectious virions. [73] The p7 deletion can be complemented in trans to restore infectivity, suggesting an essential role in the production of infectious virions. [73] Subgenomic replicons of HCV require no p7 for replication, but the roles of p7 in production of an infectious virion remains unknown. [14] It has been proposed that the HCV p7 protein is a member of a group of small proteins known as viroporins, which are involved in cation transfer across membranes and are important for virion release or maturation [24]. It has been recently shown with artificial membranes that the HCV p7 does form a calcium ion channel, and is inhibited by long-alkyl-chain iminosugar derivatives. [142] This same family of compounds has been found to be active against both BVDV and HBV. [15,47]

NS2/3

The NS2/3 protease mediates a *cis* autocleavage at the junction of NS2 a function that requires part of the N-terminus of NS3 (residues 904-1206 of the polyprotein) to carry out the cleavage. This event is essential to replication *in vivo*, as clones devoid of this activity are non-infectious (in chimpanzee models).[99] The NS2/3 proteases is an ER transmembrane protein, but it does not share any sequence homology with known proteolytic enzymes.[139] There has been a dearth of information on this protease and it is still unclear if the enzyme is acting as a cysteine or metalloprotease despite ten years of research since the first *in vitro* studies on the enzyme.[194] As this protease is required for replication, it is an attractive target for the development of specific antiviral agents.

NS2/3 protease cleavage site-derived peptides are modest inhibitors, whereas the NS4A-derived peptides (GSVVIVGRIVLSGK) were better inhibitors of autocleavage by NS2, with an IC_{50} of 0.6 μM against purified enzyme. NS4A is known to bind, activate and induce conformational changes in NS3. It was thought that the binding of NS4A and a subsequent conformational change in NS3 prior to the *cis* cleavage event makes the cleavage site for NS2 somehow inaccessible.[40] Compounds that interfere with heat shock protein chaperone activity, such as geldanamycin, were also good inhibitors of NS2/3 activity, suggesting that molecules that interfere with NS2/3 conformational changes may lead to potential antiviral compounds.[192] However, as NS2 is only involved in a single *cis* cleavage event, more attention has focused on the NS3 protease as the primary target for protease inhibitors.

NS3 and NS4A

NS3 is a bifunctional 70 kDa enzyme, whose protease activity localized to the N-terminal 180 amino acids, and the remaining two thirds of the protein encoding nucleoside triphosphatase and helicase activities. [69,70,180,198] NS3 mediates a *cis* NS3/4A cleavage, followed by *trans* cleavage of the remaining NS4A/4B, NS4B/5A and NS5A/B junctions to yield the remaining mature non-structural proteins (Figure 1-1). Although both protease and helicase activities can be expressed independently, there is little evidence the protease domain is physically separated from the helicase domain during the viral life cycle and the presence of the helicase domain may actually affect the inhibitory activity of compounds directed to the protease activity. [86] This NS3/4A heterodimer complexes with NS5B to form the replication complex in HCV infected cells. [85]

Proteinase activity

NS3 is a chymotrypsin-like serine protease that forms a tight interaction (subnanomolar) with a second viral protein, NS4A *in vivo*. [67] This high affinity interaction is though to be a co-translational event. [4,5] NS4A is a 54 amino acid residue protein that acts as an essential cofactor for the NS3 proteinase. [51] NS4A enhances the protein cleavage of all junction sites by NS3. This enhancement is upwards of a thousand fold in some assays, [102] but is absolutely required for processing the NS3/4A and NS4B/5A junctions. [52,110] The N-terminal 20 amino acids of NS4A are thought to act as a type I transmembrane domain anchor to which NS3 binds to and is thereby localized to the ER. The C-terminal 20 amino acids have no known function but possess a predominantly negative charge. Not only does the interaction with NS4A activate and localize NS3 to the ER, but it also stabilizes NS3 and increases the half-life of NS3 in the cell. [193] Immunoprecipitation studies revealed the central hydrophobic sequence of NS4A was responsible for both binding to and activation of NS3, and mutational analysis suggested that several of these residues, such as Ile-29 and Leu-31, were indispensable for this activity. [9,22,96,111,181] These findings were subsequently verified in studies with synthetic peptides and purified enzyme in cell free assays. [163]

Only a central hydrophobic domain of NS4A, residues 21-34, is required to elicit the full activation of the NS3 protease domain. [163] This domain of NS4A has a binding constant that varies with buffer conditions and between strains of HCV, but under most conditions has a Kd between 1-20 μM. [12,105] This portion of NS4A mediates most of the binding between NS3 and NS4A *in vivo*. The addition of the NS4A cofactor peptide dramatically increases the NS3 k_{cat} and k_{cat}/K_m catalytic parameters when measured against small peptide substrates representing the different site-specific junctions of the polyprotein. In particular, the NS4A peptide activates NS3 cleavage of decamer peptide substrates spanning the P_6-P_4' residues. [105]

The 3D crystal structure of the protease with and without the NS4A cofactor peptide have been reported by several groups. [92,118,196] The structure revealed that the NS3/4A complex adopts a canonical chymotrypsin-like fold consisting of N-terminal and C-terminal β-barrel domains. The residues of the catalytic triad, His-57, Asp-81 and Ser-139, are located in a space at the interface between the two domains. [41] The C-terminal β-barrel domain of the NS3 protease forms a six-stranded β-barrel as seen in other chymotrypisn-like serine proteinases, while the N-terminal β-barrel contains two additional strands, one contributed by the N-terminus of the protease and the other by the NS4A peptide. [92] This short, highly conserved hydrophobic sequence from NS4A adopts a β-strand conformation, which then intercalates in a β-sheet within the N-terminal domain of the NS3 proteinase. There is a noteable requirement for a zinc ion that can be observed in the structure whose function is the maintenance of the overall folding of the active protease. [92]

The mechanism whereby NS4A activates NS3 is still not clearly understood, however structural analyses have shown dramatic conformational changes in the first 22 N-terminal residues of NS3 proteinase domain following NS4A binding, [52,154] whereas the core of the enzyme remains unchanged upon NS4A binding. The NS3 amino terminus acts analogously to a clamp to lock

NS4A into the N-terminal β-barrel. The peptide backbone of NS4A forms extensive hydrogen bonds to the NS3 protease and several hydrophobic side-chains from NS4A are deeply buried in the hydrophobic core of NS3. The estimated buried surface area is more than 2300 squared angstroms. [92] Thus NS4A is considered an integral structural component of NS3. When the N-terminus of NS3 becomes more ordered, two residues of the catalytic triad (His-57 and Asp-81) found within the N-terminal β-barrel are able to form a hydrogen bond between the carboxyl group of Asp-81 and the His-57 imidazole. The imidazole is then polarized and can act as a general base catalyst to the catalytic Ser-139. In the absence of NS4A and this ordering effect, the carboxyl group of Asp-81 points away from His-57 and forms a hydrogen bond with Arg-155. [41,196]

One model of the NS3/4A association comes from the analysis of the structure of NS3 with and without the NS4A cofactor. [119] Based on these observations one suggested scenario for NS4A complex formation is: (1) residues 28-33 of NS4A bind NS3, (2) Glu-30 of NS3 breaks a salt bridge with Lys-68 on a double-stranded β-loop, D1-E1 (residues 62-72 of NS3), which then rotates to interact with Arg-92 of NS3 and Arg-28 of NS4A, (3) The D1-E1 loop shifts to create a complementary binding surface for the rest of NS4A (residues 21-27) and finally (4) the bulk of the N-terminus of NS3 folds from an extended structure into an α-helix (residues 13-22) and β-strand structure (residues 2-10) by interacting with the solvent-exposed side of NS4A and the enzyme surface (Figure 1-3). [118,119] This cooperative rearrangement model is consistent with the

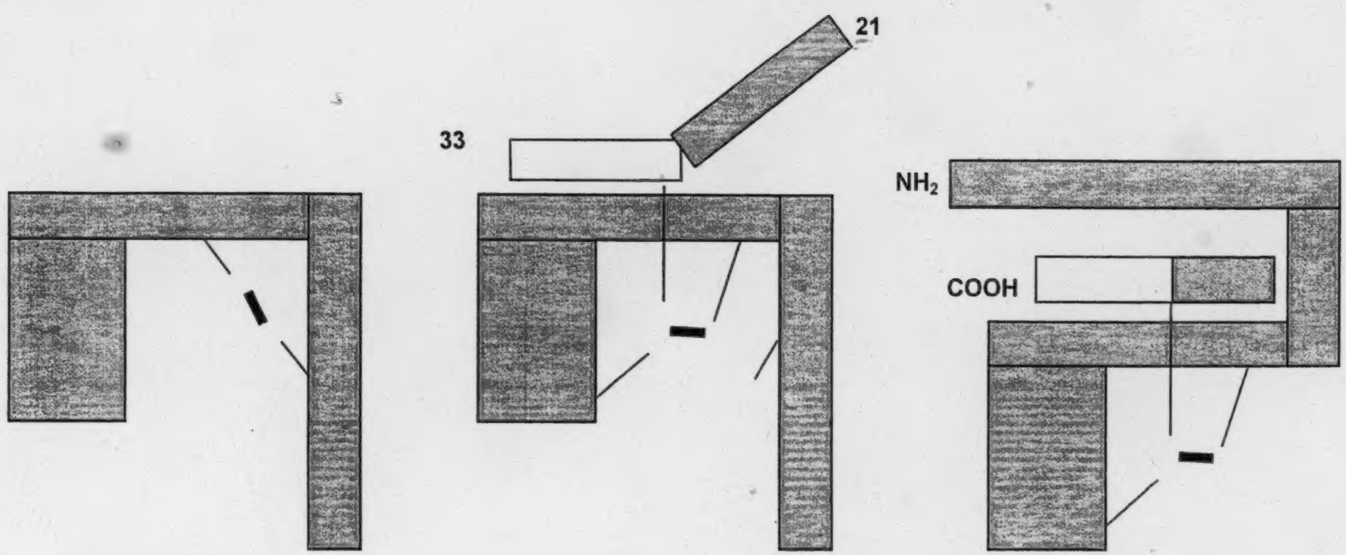

Fig. 1.3. Possible mechanism of NS4A binding to NS3. (A) There is a key salt bridge (bold line) between Glu-30 (E)and Lys-68 (K) of NS3 which forms a type of "hinge". The N-terminus of NS3 is in an extended conformation. (B) The residues 28-33 of NS4A (the white box) bind at a hydrophobic site of NS3. The Arg-28 of NS4A, together with Arg-92 of NS3 form a salt bond with Glu-30, which breaks the interaction with Lys-68. (C) The "hinge" region is now mobile and the loop on which Lys-68 resides is able to shift and creates a new binding surface for the remainder of the residues of NS4A 21-27 (the grey box). The remainder of the N-terminus of NS3 folds into its final secondary/tertiary structure.

measured slow association rates between NS3 and NS4A.[12] Interestingly, mutation of the Arg-28 of the NS4A cofactor resulted in a competitive inhibitor of NS4A activation.[163] Several groups suggested that the NS4A binding site might be a potential target for the development of antivirals, but also warned that inhibitors would have to contend with the strong, cotranslational interaction that occurs *in vivo* between NS4A and NS3.[5,143] Most of the attention for antivirals has therefore focused on the NS3 substrate-binding site.

The substrate-binding site of NS3 is shallow and entirely solvent exposed, making structure based drug design a challenge.[11] The protease prefers long peptide substrates, especially decapeptides spanning P_6-P_4'.[170] Residues distal to the scissile bond contribute significantly to binding through electrostatic interactions with a general consensus cleavage sequence of:

(Asp/Glu)-X-X-X-X-(Cys/Thr)Δ(Ser/Ala)-X-X-(Leu/Trp/Tyr)

P_6 P_1 P_1' P_4'

The P_1 residue is cysteine in all but the first intramolecular cleavage site, and is the major determinant of specificity.[70,147] The Cys appears to form a favourable van der Waals interaction with a Phe at the bottom of a pocket where the P_1 Cys sits.[92] Other requirements are negatively charged residues at P_6 and P_5, small residues in P_1' and a hydrophobic residue at P_4'.[84] It appears that several basic amino acids (Arg117, Arg119, Arg123, Arg161 and Lys165) contribute to the positive electrostatic site where the acidic residues of the substrates bind. [30] This high degree of electrostatic complementarity may compensate for the apparent lack of well-defined binding pockets seen in other cellular proteases. [11]

Molecular modelling and docking studies of a decapeptide substrate confirmed many of these predictions, particularly the substrate P1 Cys side-chain interactions with Phe-154. [102] In addition, models also suggest that the Tyr side-chain at P4' of the substrate is in close proximity to Ile-29 of NS4A. This model is consistent with Ki data of peptide inhibitors measured in the presence and absence of NS4A. [105] When the NS4A cofactor peptide was removed from the NS3:NS4A complex, the largest loss in binding was seen for inhibitors which extended to P4'. Ki data for inhibitors which extend to the prime side decreased by one order of magnitude upon removal of NS4A from the NS3/NS4A complex. [102]

One of the earliest leads in the design of HCV NS3 protease inhibitors came from the realization that NS3 is inhibited by its own cleavage products. [169] Product inhibition to the extent shown by the HCV NS3 protease is unusual. [169] Hexapeptides derived from these cleavage junctions exhibited Ki-values up to one order of magnitude lower than the Km for the corresponding substrate. It should be noted, however, that viral strains differ in their susceptibility to cleavage product inhibitors, which suggests that a proteinase inhibitor directed against one strain of HCV may not be nearly as effective against another strain. [105,169] Indeed, this has recently been shown with BILN-2061, a protease inhibitor designed by Boerhinger Ingleheim which is very effective against genotypes 1a and 1b,[104] but not against genotypes 2 or 3.

The first generation of NS3 protease inhibitors began with hexapeptides derived from the P_6-P_1 sequence (DEMEEC) of the NS4A/B junction. [84] Modelling suggested the P_1 α-carboxylate formed extensive interactions in the active site of the enzyme. Specifically, hydrogen bonds could form with the backbone amides of Ser-138 and Gly-137 (the "oxyanion hole"), with the $N^{\varepsilon 2}$ of the catalytic histidine and with the side chain of the conserved Lys-136. [143,169] If this carboxylate is deleted or amidated, there is a major loss of activity. [169] Subsequent optimization of the P5-P2 residues in a combinatorial approach lead to the discovery of the potent inhibitory peptide sequence Ac-Asp-D-Gla-Leu-Ile-Cyclohexylalanine(Cha)-Cys-OH, with an IC_{50} = 1.5 nM. [84] Later, less peptide-like product-based inhibitors were designed, mainly by Llinas-Brunet, Tsatrizos and coworkers at Boehringer Ingelheim. Truncated peptides with high potencies were obtained primarily via extension of the side-chain of a proline-based P2 residue, and P1 optimization. Cyclization, leading to a 14-membered ring, [185] has yielded the first HCV NS3 protease inhibitor to enter clinical trials. [103] These advances in protease inhibitors were made possible by first using combinatorial approaches to find lead molecules and subsequently using molecular modelling and structural analysis to fine-tune the leads.

Helicase activity

Although several crystal structures of the helicase domain have been solved, the biochemistry of this enzyme is still not well-understood. [91,197] The helicase activity is absolutely required for viral replication in the chimpanzee and is therefore another possible target for antiviral therapy. [99] The NS3 helicase is thought to unwind RNA secondary structure impeding the RNA polymerase activity of NS5B or it might unwind duplexes of positive and negative strand that may form during replication. Binding of polynucleotides dramatically increases the nucleoside triphosphatase activity of the enzyme, which is essential to the RNA unwinding activity of the enzyme. [134,148] There is currently some dispute as to whether the active enzyme is a monomer, a dimer or an oligomer. One problem noted in a review is that all of the *in vitro* experiments done with the helicase had to be performed at very low ionic strength. [152] Under these conditions, electrostatic interactions between the protein and polyanionic nucleic acid are stronger than they would be under physiological intracellular conditions. The enzyme has no appreciable activity at physiological ionic strength, either as an isolated domain or as full length NS3, which suggests that other components must be involved in helicase function. [66,67] One possible candidate, NS4A, has been shown to stimulate the helicase activity of full length NS3, but other investigators report that NS4A inhibits helicase activity. [67,80]

NS4B

NS4B is a poorly understood protein, whose function has yet to be determined. It is a 27 kDa hydrophobic protein, and its gene locus is similar to that in other *Flaviviruses* and is required for replication. Coimmunoprecipitation studies revealed an interaction between NS3, NS4A and NS4B, where NS4A mediates the interaction between NS3 and NS5B. [112] This may account for the mechanism of NS3 mediated cleavage of the HCV polyprotein, where NS4A binds NS3 and simultaneously helps recruit the NS4B-5B polyprotein, preventing the dissociation of the rest of the polyprotein (NS4B-5B) from the protease.

NS5A

Studies on the viral kinetics of HCV polyprotein processing demonstrate that the last cleavage event between NS4A and NS5A. [7] NS5A has been shown to be membrane associated in a complex with other HCV NS proteins. It is hyper-phosphorylated. It is essential, but its biochemical function is still not certain. [50] A role for this protein in blocking the interferon response has been proposed. A region in NS5A dubbed the IFN sensitivity determining region (ISDR), shown to inhibit the PKR activity induced by interferon, has been proposed to play a role in the mechanism by which NS5A inhibits the action of IFN. [65,95] Unfortunately, this function appears to be true only for the J strain of HCV and not for other strains. [176] A new putative interaction between two non-

ISDR regions of NS5A and 2',5'-oligoadenylate synthetase (2-5 AS), another important anitiviral protein has been proposed and a single mutation (F37N of the J strain NS5A protein) diminished the interaction of NS5A and 2-5 AS in cultured cells. The same mutation when introduced into the context of the replicon model was found to be highly disadvantageous for replication.[173]

Other regions of NS5A of biochemical importance are becoming understood. One report has not only demonstrated the requirement for the N-terminal amphipathic helix (AH) of NS5A in targeting the protein to the ER but that deletion of the AH region completely abates replication. The same authors showed that AH peptide-mimics inhibit the membrane association of NS5A in a dose dependent manner. This pharmacological intervention was effective against a variety of HCV isolates, suggesting that it may yield broadly effective HCV antivirals. [50]

NS5B

The other major target for antivirals is the NS5B protein, [42] an RNA-dependent RNA polymerase (RdRp) anchored to the ER membrane at its C-terminus. This enzyme is required for the production of both strands of the viral genome, and was initially recognized by a Gly-Asp-Asp motif commonly found in reverse transcriptases and other viral polymerases. [27] The full-length enzyme purified from a variety of different expression systems, [10,114] was characterized by poor catalytic activity and solubility, which hindered thorough analysis of the enzyme and the discovery of antiviral compounds.[114,116,182] Deletion of the 21 amino acid hydrophobic C-terminal membrane anchor resulted in better enzyme preparations, permitting a better biochemical analysis and the determination of several crystal structures.[56,182] The purified enzyme has been able to copy the entire HCV genome in a highly processive manner. As with other polymerases, the HCV RdRp adopts a classic "right hand" shape, with recognizable fingers, palm and thumb subdomains. The HCV RdRp is unique in that it does not adopt an "half-open right hand" architecture, but forms a more compact shape when compared with other polymerases.[18,19]

Initiation is a critical step in viral replication and the question is always how are the terminal sequences are preserved ? Some viruses, such as poliovirus, utilize a protein to act as a primer to which the polymerase adds nucleotides in a template-dependent manner. Most viral RNA polymerases, however, are capable of de novo initiation of RNA synthesis.[141] Although earlier studies with purified RdRp utilized an RNA primer in their studies of the HCV polymerase, it was more recently shown that NS5B is capable of a primer-independent mechanism of replication initiation.[122,162,201,202] In this case, there is no protein or nucleic acid primer required, only the 3'-OH from a single nucleotide triphosphate. The very recently elaborated structure of the NS5B in complex with nucleotides also supports the *de novo* initiation hypothesis.[18] The evidence suggested that that NS5B initiates RNA synthesis by a "copy back" mechanism, where the 3'-end of the RNA genome acts as a template primer for polymerization.[79] The result would be a double stranded template where the two strands are covalently linked. This would subsequently require a nuclease activity from virus (but none as yet is predicted or expected) or the host. In addition, if part of the 3'-end is required for priming, then unless there is some way to replenish the 3' end, there would be a processive loss of that sequence from the overall length of the genome. The other possibility is that a primer independent "copy back" mechanism occurs.[114] The mechanism that occurs in infected cells or not is not yet known. Current opinion is that HCV utilizes the *de novo* mechanism to initiate its replication in infected cells.[161]

Several inhibitors for the polymerase have been reported, and this is a fruitful area for the discovery of antiviral drugs for HCV. The hope is that because there is no mammalian homologue to the HCV RdRp, specific inhibitors might be found that block HCV replication with minimal cellular toxicity.[42] The only nucleoside analogue that has been shown to be useful against HCV is D-ribavirin, although this mechanism of action is currently under debate.[106] It has been suggested that the triphosphorylated form is incorporated into the HCV genome, which leads to an increased error rate of the viral polymerase. This has been demonstrated for poliovirus, which also possess an RdRp.[38] Unfortunately, ribavirin monotherapy does not decrease HCV viral titres, and an alternative mechanism for its action may be to improve liver function. Ribavirin alone is able to reduce alanine aminotransferase (ALT) levels, and dramatically improves interferon therapy when used in combination.[106] There has been much anticipation of the use of chain terminating nucleosides that have been invaluable in the treatment of other chronic viral infections, such as lamivudine for HBV. [160] Recent reports suggest that a series of 3'-deoxyribonucleotides, the most potent of which is 3'-deoxycytidine, have been found to be active against the HCV replicon in Huh 7 cells.

Other inhibitors include benzimidazole derivatives, which fall under the category of non-nucleoside inhibitors of the NS5B. One such derivative, JTK-003, is an orally bioavailable compound that is currently undergoing clinical studies in HCV patients who are non-responsive to interferon therapy. [127] Other compounds such as diketoacids, which are thought to act as pyrophosphate analogues and may chelate metal ions in the enzyme active site, are also being investigated. [74]

3' UTR

The 3' UTR which follows the polyprotein stop codon consists of a stretch of about 30 nucleotides that varies between genotypes followed by a poly(U) tract of variable length, a polypyrimidine (U/UC) stretch, and a highly conserved 98-base sequence. RNase cleavage studies and computational analysis revealed that the 3'-UTR can form stable, conserved stem-loops. [177] Much attention has therefore been drawn to the conserved 98 base sequence, as several proteins have been found to bind to it including: polypyrimidine tract binding protein, La autoantigen, hnRNP C, glyceraldehyde-3-phosphate dehydrogenase and NS3. [3,121,144,168] This is a region thought to be involved in viral replication and possibly translation. [177] Chimpanzee studies demonstrated the importance of the 3'-UTR elements, as clones deleted in the poly (U/UC) and 98 base regions, were not infectious. [99]

III. Models Systems for the Study of HCV Infection

Gaining an understanding of the replication of HCV and the testing of both neutralizing antibodies and antiviral drugs against HCV infection have been delayed by the lack of both an effective cell culture system for the virus and a suitable animal model. However, recent advances in both tissue culture and animal models promise to assist in answering important questions regarding the life cycle and pathogenesis of HCV.

Tissue Culture Models

The first generation of tissue culture models used mammalian cell lines to support the growth of HCV. Several cell lines including MT2, peripheral blood mononuclear cells, lymphocytes cell lines, and hepatocytes from humans and chimpanzees have been reported to support HCV replication. [124] Hepatocyte models are expensive and difficult to obtain or are not easy to maintain in culture for extended periods. Epstein-Barr virus (EBV) appears to enhance the replication of HCV in MT2 cells with the EBNA1 gene product thought to be important for this enhancement. [171] HCV replicates poorly in these systems and requires the use of reverse transcription-polymerase chain reaction (RT-PCR) to detect replicated viral RNA. [123] Whether or not these models represent legitimate replication is somewhat irrelevant as they were not robust enough to serve as viable cell culture models for studying any aspect of viral replication or inhibition thereof.

The second generation of tissue culture models were the subgenomic replicons. These contain the HCV-IRES driving the production of an antibiotic resistance gene G418, followed by a second IRES, driving the expression of most of the viral NS genes. [14,115] The viral structural genes are deleted in the replicon model systems. These replicons were able to replicate to high levels in a specific hepatoma cell line, and it was possible to radiolabel the viral RNA and proteins. Interestingly, the replicons contain mutations which enhance their replication in cells but prevent permissive replication in primates. [21] The most efficient replicon developed carries 3 adaptive mutations, two in NS3 (E1202G and T1280I in the Con-1 strain) and one in NS5B (S2197P) that enhance RNA replication cooperatively.[21] The mechanism for this is not known but may be an adaption to a limited cell culture factor that would be otherwise necessary for replication.[113] Moreover, data also showed that the host cell itself contributes significantly to the level of RNA replication.[100] By using a functional screening assay Huh-7 cells were identified that are up to 100-time more permissive than the original cells we used to establish the replicon system. These findings allowed the development of transient replication assays as well as cell lines carrying selectable full length HCV genomes.[145]

The replicon has become invaluable for model antiviral screening and also selecting for protease inhibitor resistant mutants.[6,183] as well as dissecting the pathways associated with IFN-γ or IFN-α interference of replication. [26,60,61] Although tumour necrosis factor alpha (TNF-α) does not appear to affect the HCV replicon[59], it does affect capsid formation of HBV, another liver-associated virus.[13] As the full reconstitution of the HCV replication cycle (including capsid formation) has not yet been achieved in cell culture, it is not yet possible to dismiss TNF-α as having no antiviral activity and one must be cautious with over-interpreting *in vitro* results.

The third possible generation of tissue culture models are derived from an HCV-infected non-Hodgkin's B-cell lymphoma and appear to provide a reproducible cell culture system for studying the complete replication cycle of HCV. In the first report of its kind, the authors were able to demonstrate the production of virions that could infect primary hepatocytes. [172] These observations clearly implicate B-cells as a potentially important reservoir for HCV *in vivo* thereby accounting for some of the HCV-associated pathologies such as mixed cryoglubuliemia. [172] Should this cell culture model be replicated by other groups and be as effective as its potential suggests, this could be an invaluable cell culture model to study the entire life cycle of HCV.

Chimpanzee Animal Model

Chimpanzee (*Pan troglodyte)* are genetically very close to humans and have been the primary animal model for studying HCV infection *in vivo*. RNA transcribed from a cloned HCV cDNA was able to initiate infection and cause a disease profile similar to that of HCV infected humans. This was convincing evidence that HCV was the causative agent for this disease. [78] Although a very expensive model, it has been used to study the RNA elements necessary for infection, and has provided a good understanding for the immune responses to this virus. [97-99]

Mouse Animal Models

There are currently two mouse models that appear capable of sustaining an HCV infection. The first model reported uses a lethally irradiated mouse reconstituted with SCID mouse bone marrow cells, and subsequently transplanted under the kidney capsule with a fragment of HCV infected human liver. The model was used successfully to demonstrate the antiviral efficacy of an HCV IRES small molecule inhibitor and an anti-HCV antibody in reducing the viral loads in a dose dependent manner. [83] However, the kinetics of HCV replication in this model are variable and the virus infection is not sustained for long periods. The second model uses an immunodeficient SCID mouse carrying a plasminogen activator under the albumin promoter (Alb-uPA) in a transgene array. As the mouse matures the toxic transgene is expressed, and there is a dramatic loss of endogenous hepatocytes. These mice can be rescued by implanting human hepatocytes which replicate to form chimeric mouse/human livers. These chimeric livers can be infected with HCV and maintain a high viral load for extended periods, making this mouse an attractive model for passive immunity and antiviral studies. [129]

IV. Combinatorial Approaches for the Discovery of Novel Antivirals

Prior to the widespread use of molecular biology, most antivirals were discovered by demonstrating inhibition of viral replication in replication assays or animal models. These screening approaches were often limited to a "one compound per well" format and therefore quite slow. The advantage of this approach was several fold. First precise knowledge of the mechanism was not necessarily required. Second any stage of the viral replication might be targeted simultaneously, therefore this might be considered a "one compound, many experiments" approach to antiviral screening. This is an extremely efficient screening approach to evaluate the potential of a single small molecule. Third the molecule would have to be cell permeable and be able inhibit its target in context of a cellular environment. Nucleoside analogues dominated the antiviral repetoire prior to 1996.

However by 1996, non-nucleoside mall molecule antivirals were being developed against other targets such as proteases as a result of better understanding of virus life cycle at the molecular level. As viral proteins can be readily produced through cloning technology, crystals suitable for structure determination could be grown, and specific assays could be configured for high throughput screening arrays. [87] Advances in both combinatorial biology and chemistry have allowed the rapid production of large numbers of compounds in parallel and significantly increased our ability to identify active compounds. The advantages of this approach are that inhibitors can be exquisitely designed for their target. The disadvantage of this approach is that an inhibitor might be developed that may not be cell permeable and possibly interact with cellular factors that might only be observed late in the development cycle of an antiviral lead drug. Regardless, the cell free assay is most useful for the majority of high throughput screening assays.

1. Why use a combinatorial approach ?

Although structural biology promises to define the details of a particular target at the molecular level, the exploitation of that information for rational drug design purposes has not yet been fully realized. The predictive power of structure-based drug design is still limited considerably by the vast number of conformations available to a protein. Thus several different structures of potential drug candidates are often proposed for testing. In this case, a combinatorial synthesis is often employed to generate the series of variants on an otherwise similar chemical backbone. The development of rationally designed protease inhibitors to HIV, such as Agenerase by Vertex Pharmaceuticals, was possible because the deep hydrophobic pockets near the active site constrained the design to small molecules which would fit into these pockets. However, the HCV NS3 protease is an excellent example of a target that lacks elements that a rational drug design approach might exploit. Early publications on the NS3 structure decried the poor prospects of using rational drug design to develop effective protease inhibitors because of the relatively shallow, solvent accessible, active site. [92] This problem suggested that an "irrational" or combinatorial approach might be the best way to develop the first generation of protease inhibitors.

There are numerous examples in the scientific literature of combinatorial approaches, and many are applied to the discovery of antivirals. A broad range of inhibitors have been found by these techniques, including enzyme inhibitors, inhibitors of protein-protein interactions and even prtein-nucleic acid interactions. In order to simplify the various approaches, an arbitrary division between biological and chemical libraries has been made in what follows.

2. Biological Libraries

A "biological" library in this case refers to natural products such as peptides, or nucleic acid sequences that employ solely the 20 naturally occurring amino acids or the standard four deoxyribonucleosides or ribonucleosides. The power of biological libraries is two-fold. First, their potential diversity is astronomical, particularly when they are not limited by cell transformation efficiencies – very large libraries of up to 10^{14} can be built *de novo*. Second, they are easy to expand by amplification, either *in vivo* or *in vitro*. These two defining factors have made biological libraries excellent methods for the rapid discovery of ligands. [53] The drawback in this case is that the products, either peptides or nucleic acids, are very susceptible to degradation by proteases and nucleases respectively and may not necessarily easily lead to a useful small molecule antiviral.

Peptide-Based Libraries

The most commonly employed biological library for the discovery of antivirals is the peptide-based library. There are several examples of peptide libraries including: aptamers, antibodies and cyclic peptides.

Peptide Aptamers

Peptide aptamers are molecules in which a variable peptide domain with affinity for a given target protein is displayed from a scaffold protein. The two most commonly used forms of this approach are the yeast two-hybrid (Y2H) and phage display techniques.

Y2H

The Y2H system originally developed by Fields and coworkers uses a target protein X, linked to a heterologous DNA binding domain (BD) and expressed as bait in a yeast test strain. [57] Concomitantly, a peptide library of random sequence (e.g. cDNA as in the original experiments), which are linked to a heterologous transcriptional activation domain (AD), is expressed as prey. If bait:prey binding occurs, the bridging of BD and AD occurs, and a transcription factor is formed. This transcription factor is then able to activate the promoter of a marker gene, which can be monitored by either growth or a colorimetric assay. One example, which used a unconstrained peptide library, fused a series of random residues to the solvent exposed C-terminus of the *Escherichia coli* (*E. coli*) thioredoxin (Trx) protein as a scaffold, in order to find an aptamer to the HIV-1 protease. Active HIV-1 protease is a homodimer. If its quaternary structure is disrupted, there is a complete loss of activity. [140] The dimerization domains consists of a layer of beta sheets, and the peptides isolated from this system were found to mimic the intercalating beta sheets. When a pair of peptides found from the screening assay were cross-linked, the two sequences were able to intercalate into the one of the subunits. Homodimerization with the other subunit was thereby prevented and the protease was inactivated.

A second example targeted the HBV core protein. A random library was expressed within the constrained active loop of the *E. coli* thioredoxin (Trx) protein. The Trx scaffold constrains the peptide, holding it in a preferred conformation for presentation to a target (Figure 1-4). One sequence designated C1-1 was found to efficiently inhibit viral capsid formation, and consequently, HBV replication and virion production. [23] The major advantage of the Y2H screening approach is that aptamers are selected *in vivo*, but the disadvantage of this approach is the isolation of false-positive clones which must be identified by secondary screening assays.

Phage Display

The other popular method for isolating peptide aptamers is the use of phage display. Phage, are viruses that infect bacterial cells, and can be engineered to present peptide libraries on the surface of filamentous phage as part of its coat protein. As such, they can be bound to an immobilized target, washed, eluted and subsequently amplified by infection of an appropriate bacterial host. The peptides can be either linear or disulphide constrained, much like the Y2H methods. One example for HCV utilized a constrained scaffold (a Kazaal-type fold) based on the human pancreatic secretory trypsin inhibitor to present a peptide library to screen for inhibitors of the NS3 protease domain. The majority of the clones selected possessed a free cysteine thiol in their constrained sequence (which is the preferred P1 residue of NS3 substrates). Modification of either of the free thiol or the constrained Kazaal-type scaffold resulted in loss of inhibition. [45]

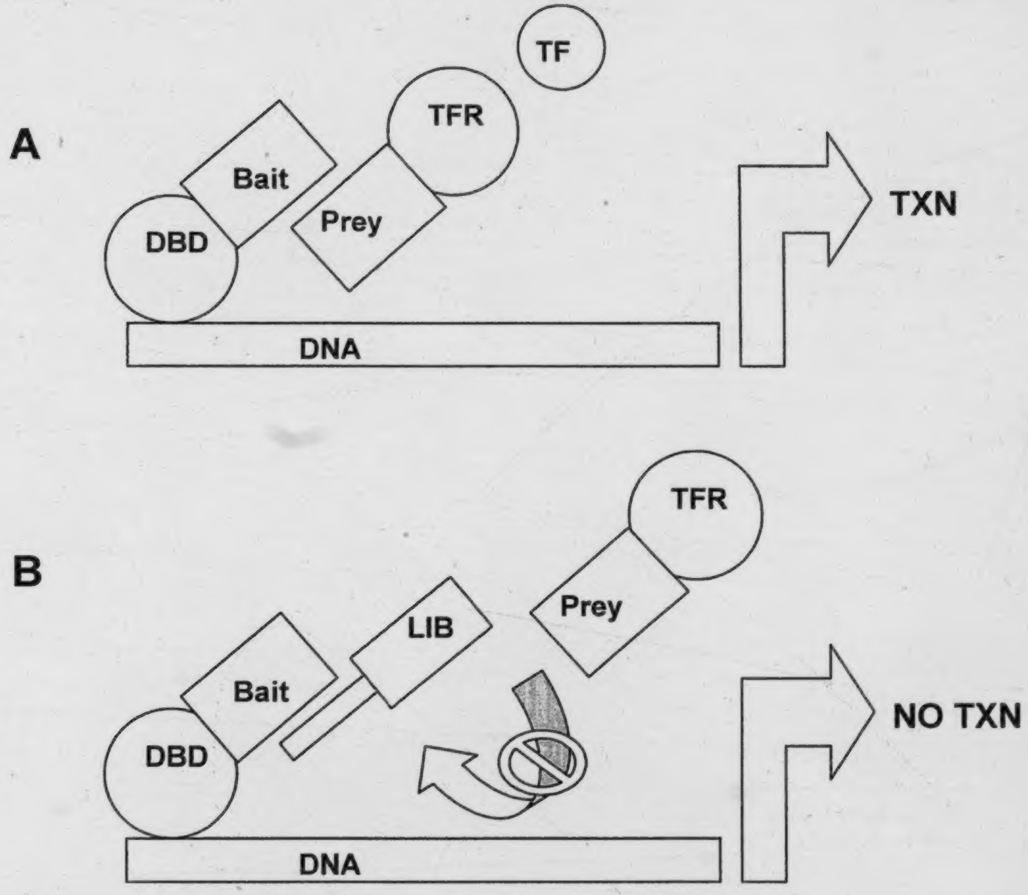

Fig 1.4. Schematic representation of the Yeast Two Hybrid (Y2H) system to find protein-protein interactions and their inhibitors. (A) The original Y2H system where a DNA binding domain (DBD) is fused with one protein (Bait), which interacts with its binding partner (Prey) which is in turn fused to a transcription factor recruiting (TFR) domain. Transcription (TXN) of a reporter or survival gene is activated and a yeast colony is selected. The identities of neither the Bait nor Prey protein fusions need be known. (B) A peptide library is fused with a carrier protein (LIB) which prevents the interaction of the Bait and Prey proteins (eg. Trx). Sequencing of the peptide appended to the carrier which prevented the Bait-Prey interaction, can rapidly isolate a useful lead for further inhibitor development.

A second and very clever example involved synthesizing the central trimeric coiled coil of the HIV gp41 which is involved in a membrane fusion event essential to viral entry. For this target however, the protein was synthesized using unnatural D-amino acids, and a decameric library phage library (of L-amino acids) was screened for binding to the target and thus identifying potential inhibitors of infection. Once the consensus sequence from the phage was determined, the mirror image of its sequence was synthesized (D-amino acids) and was found to be a good inhibitor of a natural viral infection. [49] The advantage of this approach is that the ligand is of non-natural amino acids, even though the initial library itself was "biological" in origin. Advances in peptide chemistry have made the synthesis of complete and active domains of proteins possible. [135] One example is the construction of an enzymatic active HIV protease domain that was solely of D-amino acids and was able to cleave a substrate composed of D-amino acids. These examples show the potential of this "mirror image phage display" technique for the rapid discovery of D-peptide ligands for drug development. The advantage of these peptides is they are metabolically stable, unlike their natural counterparts. It is somewhat surprising that there are not more reports utilizing this approach to antiviral discovery.

Antibodies

One excellent example of the use of antibodies as starting points for drug design involved screening a library of hybridomas for binding to the HCV NS3, followed by single cell cloning by limiting dilution. [187] One antibody, 8D4, was found to be a competitive inhibitor (Ki = 39nM) of a common decameric substrate. The inhibitory activity was reduced by the presence of the cofactor NS4A peptide, which suggests a possible conformational or steric clash upon NS4A binding. Remarkably and counter to many expert opinions that the *cis* cleavage (i.e. NS3/4A) would be difficult to inhibit, the monoclonal antibody (MAb) 8D4 showed marked inhibition of this processing event, but little effect on the NS3/4A dependent *trans* processing of the NS5A/5B polyprotein in vitro. By using a cell-surface display assay of peptide libraries a consensus epitope of the MAb was found to match the $DQD_{81}LV$ sequence in NS3 containing the catalytic site residue. This may explain why it was competitive with substrate. [187]

As most of the diversity and the majority of binding of antibodies to their targets is mediated through their six complementarity-determining regions (CDRs) the authors of the above study reasoned that sequences derived from high affinity CDRs may provide a template for designing lower molecular weight lead molecules. Peptides prepared from this region were found to be moderate (Ki ~ 40 μM) inhibitors of NS3. [186] Cyclization of the peptides through disulphide bonds improved activity some four fold, suggesting that increasing the rigidity of the conformation of the peptide improved binding. Furthermore, an inhibitory antibody to the NS5B polymerase has also been reported which may also aid in development of novel small molecules inhibitors of this target. [132]

Cyclic Peptides

From the outset, a backbone (head-to-tail) cyclized peptide library sequence has two major advantages over other peptide library screening methods. First, the structure is rigidified by its cyclization, which restricts its conformational space and reduces the entropic cost of receptor binding. This typically leads to a ligand with much higher affinity than its linear counterpart. Secondly, although the other screening methods can employ constrained peptides (e.g. disulfide bridges between cysteines), these are not metabolically stable as are the backbone cyclized peptides, and thus must be further modified to retain their rigid conformation. [159] As many pharmaceutically important natural products are based on cyclic peptides, such as the orally available 11-residue immuno-suppressant cyclosporin, this may become a valuable approach for library screening.

The most successful approach, dubbed SCICLOPPS, involves a split-intein method, and is analogous to RNA splicing which generates a lariat structure. The mechanism involves a series of intramolecular trans-splicing reactions, and has been shown to generate short cyclic peptides in both *E. coli* [159] and, more recently mammalian cells. [93] The latter study utilized a modified retrovirus to efficiently deliver and select peptides for their ability to inhibit interleukin-4 signalling in B-cells. These single cell based approaches require a selection method, thus either the use of cell survival, or some fluorescent marker that can be used by a fluorescent activated cell sorter (FACS) as a means of selection is necessary. This approach, owing to the advantages of the initial library "hit" (ie. stability and affinity), will likely see greater general application in the future, especially in HCV with the recently developed FACS assay for replicon activity. [136]

Nucleic Acid Libraries

Several different types of nucleic acid libraries have been used to study HCV, in particular, ribozymes and nucleic acid aptamers. These nucleic acids can be RNA, DNA or nucleic acids chemically altered to increase their stability.

Ribozymes

The use of ribozymes libraries to find cellular factors necessary for the HCV IRES dependent translation using reverse genomics has been recently reported. [101] The hybridizing arms of a hairpin ribozyme were randomized in a retroviral vector ribozyme library and used to infect HeLa cells stably expressing a bicistronic selection message. Positive selection involved the cap-dependent translation of the hygromycin B phosphotransferase gene. This also ensured that a given ribozyme sequence minimally affected host protein translation. Negative selection involved the translation of the herpes simplex virus thymidine kinase (HSV-tk) gene under the HCV IRES. The HSV-tk is known to initially phosphorylate the drug gancyclovir, which, following its phosphorylation by host kinases to a nucleotide triphosphate becomes toxic in HSV-tk expressing cells.

In this case only a ribozyme sequence that inhibits the HCV IRES, thereby rendering cells resistant to ganciclovir (as no protein is made to phosphorylate and activate the drug), but retains hygromycin resistance is selected. Sequencing the hybridizing arms of the ribozyme and matching the consensus sequences against the human genome demonstrated a functional requirement for two of the subunits of eIF2B (eIF2γ and eIF2Bγ). There is current interest in pharmaceutical companies to screen for small molecules that can interfere with the association of the HCV IRES RNA with the one or both of the subunits of eIF2B.

RNA Aptamers

RNA aptamers are the nucleic acid counterpart of peptide aptamers. However they can be selected and amplified with purified polymerases in a cell free manner because they require no peptide synthesis termed SELEX (systematic evolution of ligands by exponential enrichment). RNA aptamer complexity is enormous, for example pools of 4^{30} random RNA sequences can be screened for binding to a given target. There are numerous examples of such aptamers that are active against a wide variety of viral targets. For example aptamers that inhibit HIV packaging by inhibiting the nucleocapsid association or HIV reverse transcriptase (RT) have been reported. [64,81] In the case of HCV there are two major targets, NS5B and NS3. Interestingly, the aptamers discovered for both targets were potent (Kd low nM, Ki about 10 fold higher) non-competitive inhibitors and directed towards basic solvent exposed surfaces on each enzyme. These aptamers were found to require the same cluster of positively charged residues that contribute to the binding of the P_6 acidic acid residue of the cleavage junctions.[81] Although it is difficult to see these polyanionic compounds being used directly as antivirals, they may provide insight into vulnerable viral target regions and starting points for the design of anti-HCV drugs.

3. Synthetic Libraries

Synthetic libraries offer several distinct advantages over biological libraries. Firstly, they are not restricted to the use of naturally occurring amino acids or nucleotides, and the various enzymes needed to generate them. Thus their potential diversity is greater than that of biological libraries. Secondly, synthetic compounds are more likely to be resistant to metabolic breakdown and are therefore more "drug-like". The greatest challenge in the case of synthetic libraries is their screening, which can done either by solid phase or solution phase approaches.

Solid Phase Selection of Synthetic Libraries

In this case members of the library are spatially separated from one another since each member remains attached to the solid phase resin bead upon which it is built. This is the "one bead, one compound" method that is often employed in "split-and-mix" type routines (Figure 1-5). This allows use of diverse chemistries that are compatible with the resin to generate diverse libraries. The creative questions then become "what to screen" and "how to screen it" ? To show the diversity of this approach, two different viral targets are described, one characterized by an RNA-protein interaction and the other by a protein-protein interaction.

The transcription of HIV mRNA is greatly enhanced by the HIV Tat protein, which is known to bind an HIV RNA structural element Tar, and to recruit cellular factors. The contact regions of this interaction have been well mapped and a trinucleotide bulge in the stem-loop of Tar is required for Tat recognition. Exploiting this observation, a library of tripeptides consisting of D- and L-amino acids was generated [82]. As RNA is polyanionic, non-specific electrostatic interactions with positively charged amino acids on the beads giving rise to false positives was a concern. To overcome this, an excess of unlabelled Tar RNA lacking the critically important trinucleotide buldge was used as a non-specific competitor together with fluorescently tagged Tar RNA containing the bulge. Upon mixing, only individual beads that specifically bound the labelled RNA possessing the trinucleotide bulge were selected. The peptide sequence was subsequently determined and from this sequence, several lead compounds were obtained. Several isolated tripeptide sequences were subsequently shown to penetrate cells and downregulate HIV transcription *in vitro*. [82]

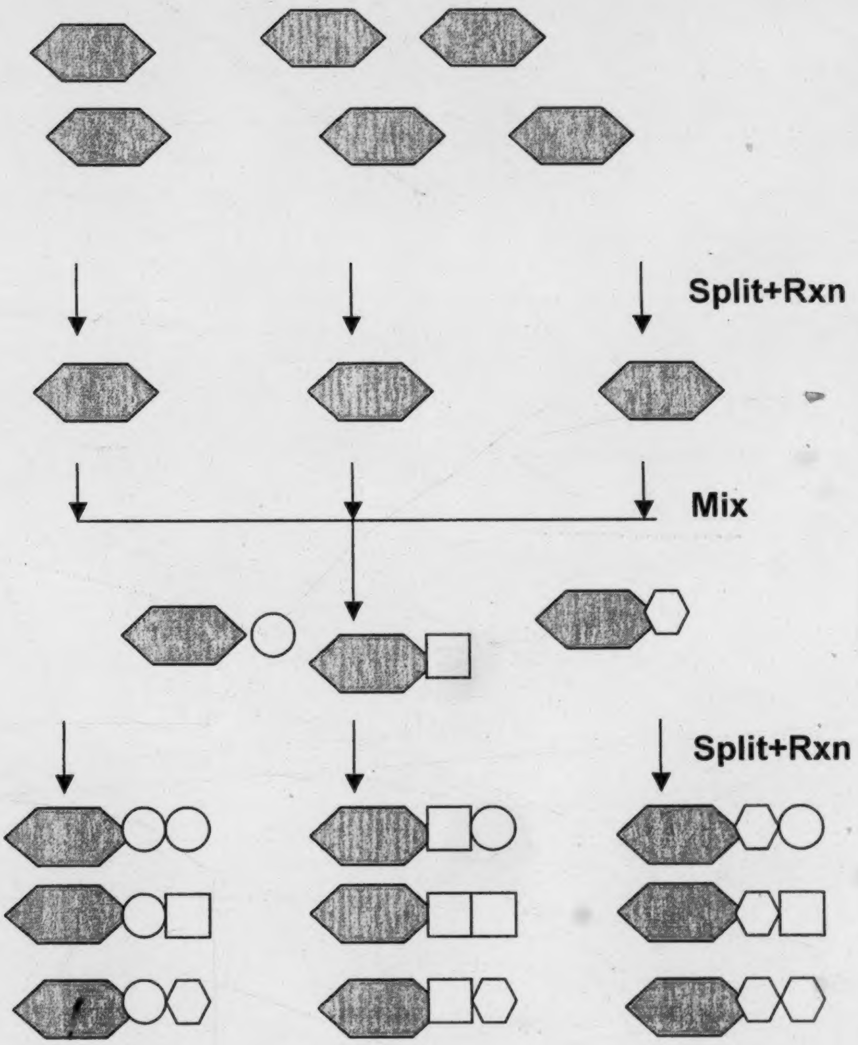

Fig. 1-5. The "Split and Mix" approach to combinatorial solid phase synthesis. The solid coloured heaxagons represent solid phase resins. The resins are randomly separated and peptides, represented by the Euclidian shapes, are chemically coupled to the resins in separate reactions. The resins are then mixed together and then randomly split for a second peptide synthesis step. The numberl of different peptides attached to the resins grows exponentially with each split and mix step. (Rxn = for example, a peptide synthesis step)

The second example involves the selection of gp41-mediated HIV-1 cell entry inhibitors.[203] The trimeric, α-helical coiled-coil of the HIV-1 gp41 ectodomain is thought to be part of a transient, receptor-triggered intermediate in the refolding of the envelope glycoprotein into a fusion-active conformation. A biased library of 61275 potential ligands was screened for binding to a biotin labelled gp41 inner core. A directed library is one which uses a limited number of building blocks chosen on the basis of pre-existing information such as structure. Selection of beads that bound the biotinylated envelope were readily identified by adding a streptavidin-alkaline phosphatase, which reacted to produce an insoluble blue dye in the ligand-carrying beads that had taken up the gp41 inner core.[203] Several labour intensive deconvolution steps (Figure 1-6) were necessary to determine the optimal sequence for binding the gp41 ectodomain, however the final isolated peptide was a potent inhibitor of cell-cell fusion. The inhibition of a complex (i.e. multiprotein, conformationally driven) event such as cell-cell fusion by ligands discovered by combinatorial methods clearly demonstrates their power in antiviral drug discovery.

The one limitation of this approach is that a single bead must be selected and further manipulated, thus there is a minimal bead size that can be used. In addition, there must be sufficient material on the bead, or some form of encoding, to allow for the determination of exactly what is on the bead. This in turn causes the weight and volume of beads used to become exponentially larger as the size of the library increases.

Deconvolution of Position	**1**	**2**	**3**
	E-Xxx-Ooo	Y-E-Ooo	Y-E-E
	R-Xxx-Ooo	Y-R-Ooo	Y-E-R
Peptides	P-Xxx-Ooo	Y-P-Ooo	Y-E-P
	Y-Xxx-Ooo	Y-Y-Ooo	Y-E-Y
	S-Xxx-Ooo	Y-S-Ooo	Y-E-S
Most active hit	Y-Xxx-Ooo	Y-E-Ooo	Y-E-S

Fig. 1-6. Example of the deconvolution of a tripeptide. In step 1 the peptides are spatially separated and a known amino acid is coupled to the terminus of the randomized dipeptide. The Xxx and Ooo represent a pool amino acids that were coupled in the two previous steps of the peptide synthesis. In step 2, once the terminal amino acid is known, the peptide is resynthesized randomizing only the C-terminal position, spatially separating the peptides to determine the best amino acid for the second position. All peptides have the Y amino acid at the N-terminus. The process is repeated for step 3. Thus in 3 steps a total pool of 5^3 or 125 peptides were screened. However, the selection strategy involves 15 separate assays to determine the most active residue.

Solution Phase Screening of Libraries

There are two basic methods for screening large numbers of potential ligands in solution phase. One involves the use of massive parallel syntheses and subsequent iterative deconvolution analysis (Figure 1-3). This approach has been used successfully to find a wide variety of inhibitors against such viral targets as the HCV NS3 protease [84] and a retroviral integrase protein. [149] The approach is however, labour intensive. In the combinatorial optimization of the Ac-DEMEEC-OH peptide as an inhibitor against the NS3 protease for example, the authors made 81 mixtures of 81 peptides to optimize just two of the residues. [84]

A second screening method uses an affinity-based method to resolve mixtures of candidate compounds. One of the most powerful methods for screening these mixtures in solution has been the application of mass spectrometry (MS) to identifying lead compounds either directly or indirectly. The most prevalent use of MS has been the indirect method – the "capture and release" approach. There are three steps to this process: (1) binding of a ligand within a pool of potential ligands to a target; (2) the separation of the bound ligand from the unbound pool of ligands; and (3) identification of the bound ligand by MS. Several examples illustrate the use of this "capture and release" approach against a variety of viral targets.

One example involves the incubation of the human cytomegalovirus protease with a panel of inhibitors under native conditions including the known inhibitor. [165] A size exclusion spin column was used to exclude high molecular weight species and trap low molecular weight compounds. If a compound binds the protease, the compound will be present in the high molecular weight excluded fraction. The excluded fraction was then treated with a denaturing solvent to release the inhibitor, and respun through a microconcentrator. The filtrate was then analysed by mass spectrometry to identify the ligand. A similar approach was used to find compounds that bind the picornovirus capsid protein. In this case compounds were added to whole viruses, the mixture was applied to a size-exclusion column and the hydrophobic small molecules extracted using a denaturing organic solvent. The organic phase was dried down and ligands were identified by mass spectrometry (Figure 1-7). A library of compounds were screened and the assay found ligands targeted for the VP1 pocket of the picornovirus capsid. [184] The "capture and release" method is attractive in that the initial binding step with the pool of ligands theoretically can be done in any desired biological buffer. This ensures that the target is biologically active, or at least that the binding event occurs in a biologically relevant buffer system. Secondly, if the target is immobilized it may be possible to include a washing step and/or to add another protein such as bovine serum albumin (BSA), to reduce the amount of non-specific binding that occurs. The presence of false positives in assays that screen mixtures of ligands is always of significant concern. [157] Alternatively, if the target is of sufficiently different molecular weight

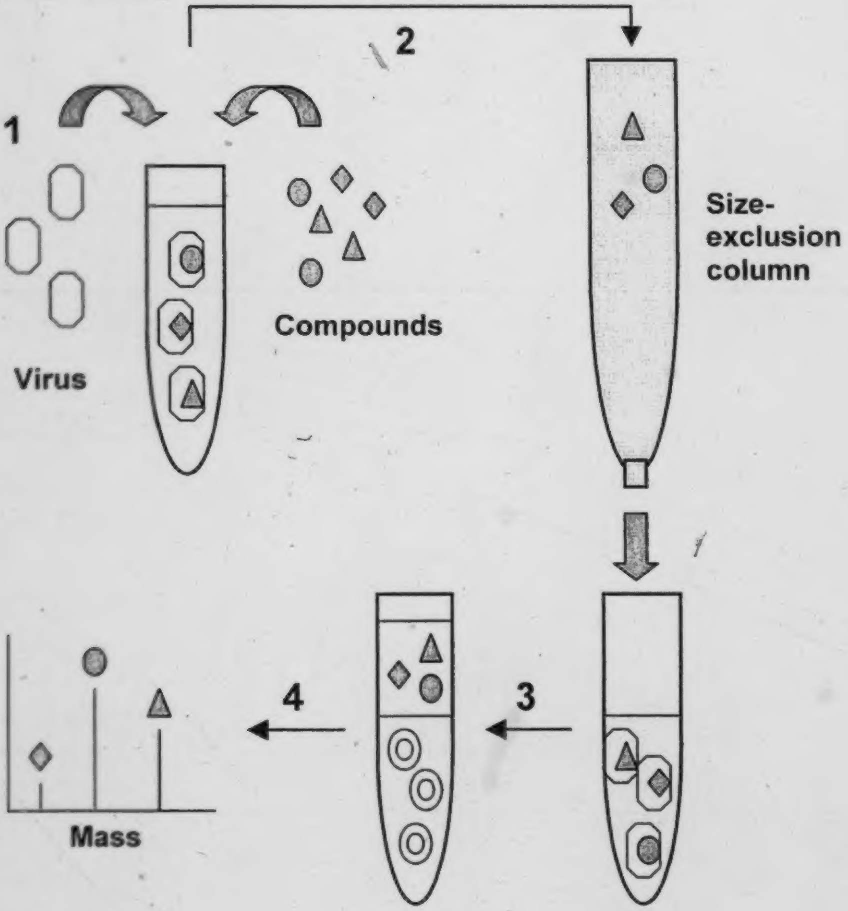

Fig. 1-7. An example of the capture and release approach to library screening using mass spectrometry. The steps are: (1) a library of compounds is added to whole viruses, (2) binding and non-binding compounds are separated by size-exclusion chromatography, (3) high molecular weight complexes are collected and then the hydrophobic compounds are extracted with an organic solvent that also denatures the virus, and (4) the organic layer is concentrated and analyzed by mass spectrometry to identify the compounds that bound the whole virus.

from BSA, it may be possible to separate the two by size exclusion chromatography.

An alternative to the "capture and release" approach is the recently developed technique of frontal affinity chromatography coupled on-line to electrospray mass spectrometry (FAC/MS) to analyze mixtures of compounds. FAC/MS is similar to conventional affinity chromatography in that a receptor is immobilized on a suitable support and packed in a column. However in frontal chromatography the sample is applied continuously to the column as opposed to the single spike in conventional affinity chromatography. Since applying a ligand continuously consumes more material than a single "spike", miniaturization of the assay is essential to reduce the amount of sample needed. Furthermore, conventional chromatography uses a non-specific detector (eg. UV absorbance) that simply reports the presence of a UV absorbing molecule but is unable to determine the identity of the absorbing molecule. A secondary analysis is needed to determine the identity of component(s) eluting at a given time (fraction). The advantage of using a mass spectrometer is that it is both sensitive and is able to identify multiple compounds in a mixture simultaneously; thus it fufills the requirement for miniaturization and provides a method of screening mixtures based solely on their elution times on a column (Figure 1-8).

FAC/MS is also capable of quantifying binding affinities in a single run. The column capacity, Bt, which is the amount of immobilized target that can bind

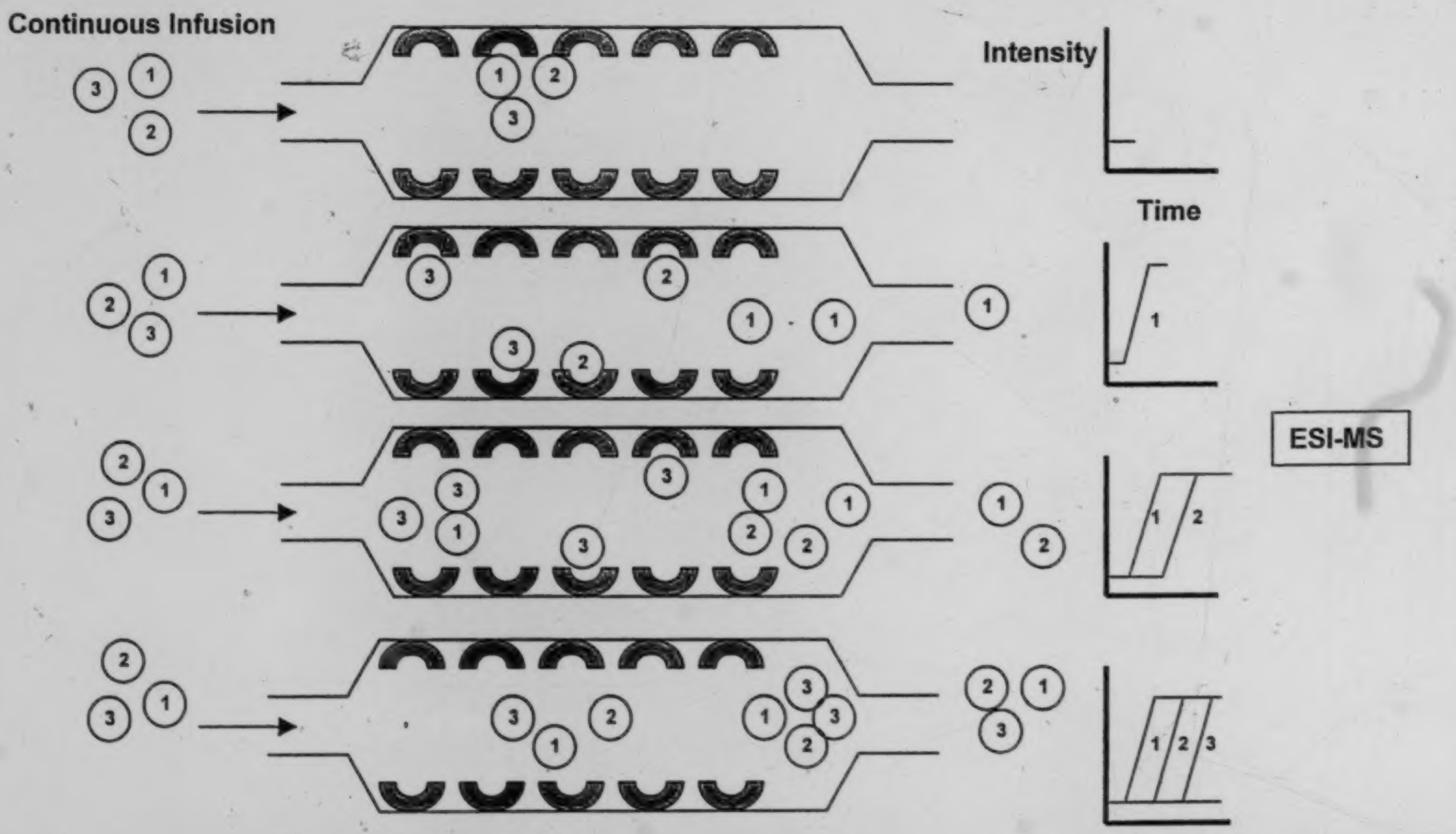

Fig. 1-8. Idealized schematic representation of the FAC/MS approach. The sample mixture is applied continuously rather than as a "spike". Compounds elute on the basis of their affinity for the immobilized target as sigmoidal wavefronts. Weakly binding compounds, as exemplified by the sphere (1) elute first, followed by stronger, and stronger ligands spheres (2) and (3). The binding affinity can be quantified by well-established theory. The column can be recycled for multiple rounds of screening as the buffer must be compatible with the biological activity of the immobilized receptor. The receptor is typically immobilized on a streptavidin coated solid support using a biotin tag introduced either through chemical or enzymatic biotinylation. In this diagram, sphere (1) would be the void volume marker, sphere (2) the weak ligand and sphere (3) the strong ligand.

ligand, can be determined using established frontal affinity chromatography theory.[89] The Bt of the column, the dissociation constant (Kd), the concentration ($[X]_o$) and the retention volume (V_x-V_o) of a given ligand are related by the following equation:

$$V_x - V_o = Bt / (Kd + [X]o) \qquad \text{Equation 1}$$

Thus FAC/MS can screen libraries of compounds and simultaneously determine the relative Kds of the individual library members in a mixture. These two features make it an attractive approach for screening and potentially quantifying the binding constants (if concentrations are accurately known) of mixtures of compounds. FAC/MS has been used to screen a variety of ligands, including carbohydrates and peptides, for a variety of immobilized targets including antibodies, lectins [199] and enzymes such as glucosyltransferases [200] and proteases.[157] Although not yet utilized in FAC/MS, other non-proteinaceous targets such as nucleic acids might be immobilized to generate affinity columns as well. However, in contrast to the "capture and release" approach, because the analysis is on-line with the chromatography step, the buffers must be compatible with the ESI-MS, as there is no extraction step to remove buffer components that might affect MS sensitivity.

Rationale and Objectives

There are numerous reports in the literature detailing various genetic and cell free methods for screening protease inhibitors for the HCV NS3 protease, however all these assays are of the "one compound per well" format. This limits the power of a combinatorial approach, as any synthesis to generate a library is inevitably forced to revert to massive parallel syntheses. The power of the combinatorial approach would be greatly enhanced by synthesizing mixtures of compounds in "one pot", and screening the mixture en masse. It was my desire to use the recently developed technique of FAC/MS as a novel assay to screen a mixture of compounds for potential inhibitors of the HCV NS3 protease.

The objectives of this project were to:

(1) Test the ability of the FAC/MS system to screen a mixture of compounds with a model serine protease, bovine thrombin (Chapter 2).

(2) Clone the HCV NS3 with an *in vivo* biotinylation tag and demonstrate the ability of FAC/MS to screen a model P1 combinatorial library derived from the NS4A/B cleavage junction (Chapter 3).

Considerable attention has also focused on developing inhibitors of viral protein-protein interactions as potential antivirals owing to their selectivity and overall importance to viral replication. One novel application of FAC/MS may be to screen minimal binding domains of viral protein-protein interactions, and possibly optimize the binding of such a domain with libraries of substitutions. Therefore, my additional objectives were:

(3) To delineate a minimal binding sequence of the central binding and activating domain of NS4A and to screen combinatorial libraries of the truncated sequence for improved binding to NS3. Such peptide sequences might bind but not activate the NS3 protease (Chapter 4).

(4) Design a bivalent peptide by linking the NS4A activating sequence (GSVVIVGIVLSGK) with a known potent substrate-based NS3 protease inhibitor sequence to improve the potency of the substrate based inhibitor. The two peptides would be linked together by a peptide sequence known to cross cell membranes (Chapter 5).

There is considerable interest in our laboratory with respect to HBV antiviral drug development and resistance, particularly with respect to the nucleoside analogue Lamivudine (3TC). Prior to beginning my work on the HCV protease, I worked on the stability of a lamivudine-resistant mutant *in vivo*. This work and a model to explain both the slow onset of lamivudine resistance in chronically patients and the rapid onset of resistance in a transplant patient is presented as Appendix I as it was clearly a separate project, but also resulted in a publication during my time as a graduate student in Dr. Tyrrell's laboratory.

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Chapter 2

Bovine Thrombin as a Model Protease for Frontal Affinity Chromatography and On-line Mass Spectrometry

1. Introduction

Proteases are ubiquitous in biology and are often targets for therapeutic intervention. One medically significant protease target for inhibitor development is thrombin, a serine protease that is involved in the signalling cascade of proteolytic events ultimately leading to clot formation. Thrombin acts by cleaving fibrinogen into fibrin (Figure 2-1). It is has been a well studied target for drug intervention, particularly in diseases where clots are formed in the absence of blood vessel trauma or where excessive clotting occurs in response to trauma.[10] There are a number of different inhibitors for thrombin including heparin, which binds soluble thrombin, and peptidomimetic substrate inhibitors such as D-Phe-Pro-Arg (fPR).[3]

With a known small molecule inhibitor, fPR, and commercially available bovine thrombin, this protein is an excellent model system to demonstrate the validity of the FAC/MS assay to screen for ligands directed towards a protease target. As thrombin cannot be immobilized in its native state, an affinity tag must

Fig. 2-1. Thrombin and its inhibitors. (A) The role of thrombin in clot formation. Activated thrombin cleaves fibrinogen to fibrin which polymerizes. Bound thrombin remains enzymatically active in the clot. Factor XIII is activated, which catalyzes the formation of the matrix of proteins, forming the clot. Platelets are subsequently cross-linked with fibrinogen. (B) Thrombin inhibitors. Thrombin has two distinct binding sites apart from the active site. Exosite 1 – helps position the fibrinogen correctly in the active site and interacts with platelets. The anticoagulants of hirudin bind here. Exosite 2 – the heparin binding site. Formation of a complex between heparin and antithrombin greatly increases the thrombin inhibitory action of thrombin. Heparin is therefore not considered a direct inhibitor. Shown in bold is a bivalent inhibitor with the fPR active site inhibitor and modified Hirudin sequence via a linker sequence: Ki = 37pM.[9]

be introduced to it in order to generate an affinity column. One of the simplest tags to introduce onto a protein for binding to the column is biotin. This is typically accomplished by using activated esters of biotin that can be randomly introduced to either ε-amino groups on lysines, or onto the thiol moiety of cysteine residues. Unfortunately, active site residues sometimes contain these functionalities. To preserve enzymatic/binding activity low molar ratios of biotinylating reagent and inclusion of a high concentration of a ligand that protects the active site are often used in biotinylation reactions.[7] For example, in the case of thrombin, leupeptin was used to protect the active site. Using FAC/MS and established frontal chromatography theory, the Kd of fPR was determined.[5] In addition, FAC/MS was also able to separate fPR from the isomer PfR, which is not a ligand for thrombin.

2. Materials and Methods

2.1 Reagents

Thrombin and leupeptin were purchased from Sigma (Oakville, Ontario). The biotinylation reagent sulfosuccinimidyl 6-(biotin-amido) hexanoate (sulfo-NHS-LC-biotin) was obtained from Pierce (Aurora, Ontario). Peptides fPR and PfR were obtained from the Alberta Peptide Institute (Edmonton, Alberta), and the void volume marker, β-D-GlcpNAc-(1→2)-[β-D-GlcpNAc-(1→6)]-α-D-Manp(1→6)-β-D-Glcp-*O*-octyl (OGMGG) was provided by the Hindsgaul laboratory. Microconcentrators were purchased from Amicon (Toronto, Ontario).

Polyetheretherketone (PEEK) tubing was purchased from Fisher (Toronto, Ontario) and streptavidin coated glass beads (Mean pore size, 1142 angstroms; particle size, 37-74 microns; capacity, 1.85 μmole biotin/g beads) from CPG (Lincoln Park, NJ).

2.2 *Random Chemical Biotinylation of Thrombin*

2.34 mgs of purified bovine thrombin as a citrate salt (Sigma) was dissolved in 500 μL of bicarbonate buffer (50 mM $NaHCO_3$, 150 mM NaCl, pH 8.54) and 34.8 μL of a 5 mM stock solution of leupeptin was added for a final ratio of 10:1, leupeptin:thrombin. The solution was rotated at 4^0C for 40 minutes. A fresh 1.8 mM solution of NHS-LC-Biotin in deionized water was prepared, 48.3 μL of which was added to the thrombin and leupeptin solution at a final ratio of 5:1, biotinylating agent:thrombin. The solution was rotated for 2 minutes at room temperature and then incubated on ice for 2 hours. To terminate the reaction, a 20 μL solution of 5 mM Tris, 15 mM NaCl (Sigma), pH 8.0, was added to the ice cold solution for 15 minutes. The reaction mixture was then spun at 4^0C for 34 minutes at 13200 rpm in a benchtop microfuge through a 10 kDa microconcentrator. The concentrate was washed with 100 μL of PBS buffer and spun an additional 5 minutes at 13200 rpm, to ensure the removal of any unconjugated biotinylating agent. The concentrator was inverted and 200 μL of PBS solution was applied to the top chamber and the unit spun for 5 minutes at 3000 rpm. This was repeated two additional times.

A micro-scale affinity column was prepared by packing streptavidin-coated beads into PEEK tubing (i.d., 0.5 mm; length, 50 mm). The biotinylated thrombin was diluted by adding 1mL of PBS and infused through the column at 4 μL/minute. Any unoccupied biotin-binding sites were blocked by washing the thrombin affinity column with a D-biotin solution (1mL, 0.2 mgs/mL in acetate buffer). After blocking, the column was washed with acetate buffer and stored at 4^0C.

2.3 *FAC/MS instrument*

The FAC/MS apparatus was set up as previously described.[7] Three one mL syringes were set up in parallel on a multisyringe pump (Harvard PhD 2000) at a flow rate of 8 μL/min, with a T-junction switching valve (Figure 2-2) connecting either the wash buffer (10 mM NH_4OAc, pH 7.4, 0.1mM calcium acetate [$Ca(OAc)_2$]) or analyte (wash buffer containing diluted ligand) to the column. The mass spectrometer compatible make-up buffer, 98% acetonitrile (ACN): 2% acetic acid (AcOH), was mixed with column eluent and introduced directly into a Hewlett-Packard 1100 MSD single quadrupole electrospray ionizer mass spectrometer (ESI-MS). The input rate into the mass spectrometer was 16 μL/min (Figure 2-2).

For characterization of the column eluent, the spectrometer scanned from *m/z* 100-1500 in 1.5 seconds in the positive-ion mode. For screening individual

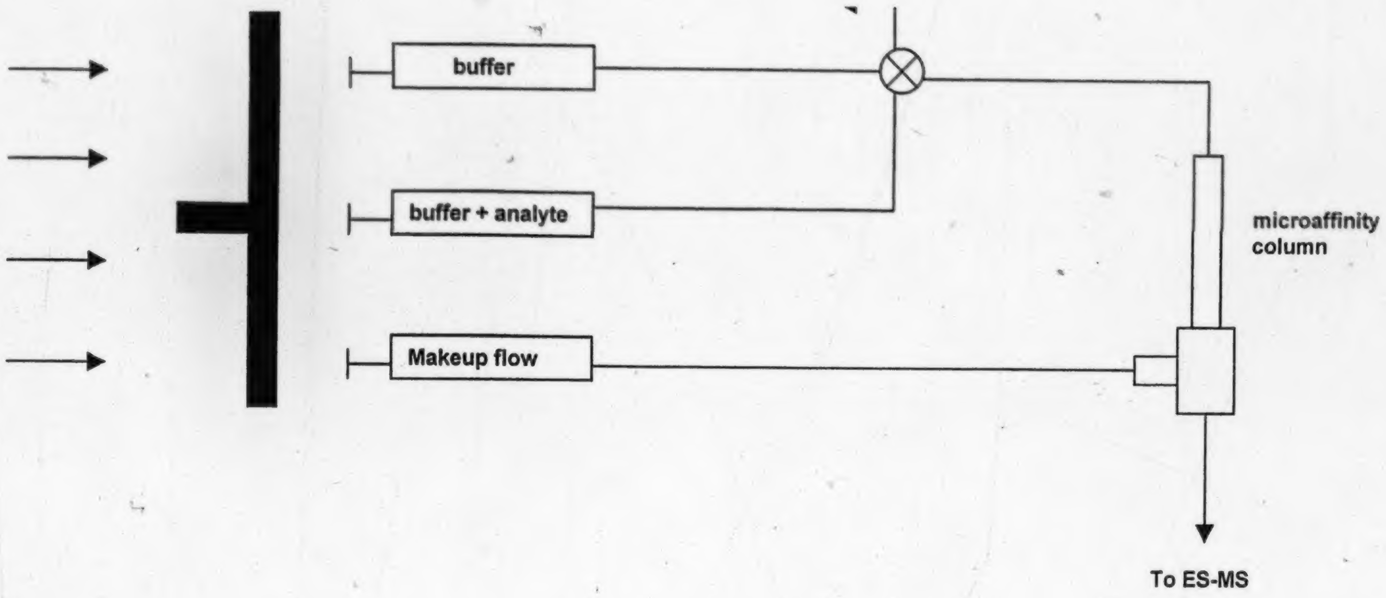
buffer
buffer + analyte
Makeup flow
microaffinity column
To ES-MS

Fig. 2-2. Schematic representation of the FAC/MS apparatus for monitoring molecular interactions. There are three independently driven syringes. Running buffer and analyte solutions were connected to the column through an Upchurch six-port injection valve. Make-up flow was introduced immediately through a mixing tee, with the combined flow introduced into an electrospray mass spectrometer.

compounds, the spectrometer was operated in selected ion monitoring (locked on the *m/z* values of the individual ligands) in the positive-ion mode. The peptides fPR and PfR were measured as their monoprotonated adducts ($M+H^+$) and OGMGG was measured as its singly charged sodium adduct ($M+Na^+$). A chamber voltage of –4000 V with a grounded electrospray needle, N_2 drying gas flow rate of 4 L/minute, and N_2 nebulizer pressure of 480 millibar were used. Breakthrough volumes were measured as midpoints in the extracted ion chromatograms.

2.4 *Measuring the Binding Constant of fPR to thrombin*

A series of solutions containing varying concentrations of fPR (1-10 μM) and a fixed concentration of the void volume marker OGMGG (1 μM) were prepared and used to perform the FAC/MS assay. The Bt and Kd values were calculated from linear regression analysis of retention volumes as a function of substrate ligand concentration using Microsoft Excell software and Equation 1. Frontal chromatograms are presented as IGOR (Wavemetrics) program files.

3. Results

3.1 Kd of fPR and column Bt

The elution time of a ligand is dependent on the column capacity (amount of active receptor), Bt, the affinity of a ligand to its receptor, the Kd, and the concentration of the ligand. A strong ligand will elute later than weak-binding or non-binding compounds. This is illustrated in Figure 2-3, where the known ligand, fPR elutes at around 8.5 minutes (V-Vo = 42.68 μL), whereas the void volume marker, OGMG elutes at around 3 minutes. Furthermore, as the concentration of a ligand decreases, its elution time increases (V-Vo = 83.25 μL) (Figure 2-4). The inverse relationship of elution time and concentration, based on equation 1, allows the determination of both the Kd and Bt of a column from the same plot. The data collected from a series of concentrations is given in Table 1-1 and shows how the data is analyzed to determine the Kd and Bt. The graph of $1/(V-V_0)[X_0]$ vs. $1/[X_0]$ (Figure 2-5) is analogous to a Lineweaver-Burke plot. The Bt is the reciprocal of the y intercept, and the Kd is determined from the slope divided by the y intercept. The activity of the thrombin column was verified by the fact that it was able to detect differences in the elution times between the isobaric diasteriomers, fPR and PfR (Figure 2-6).

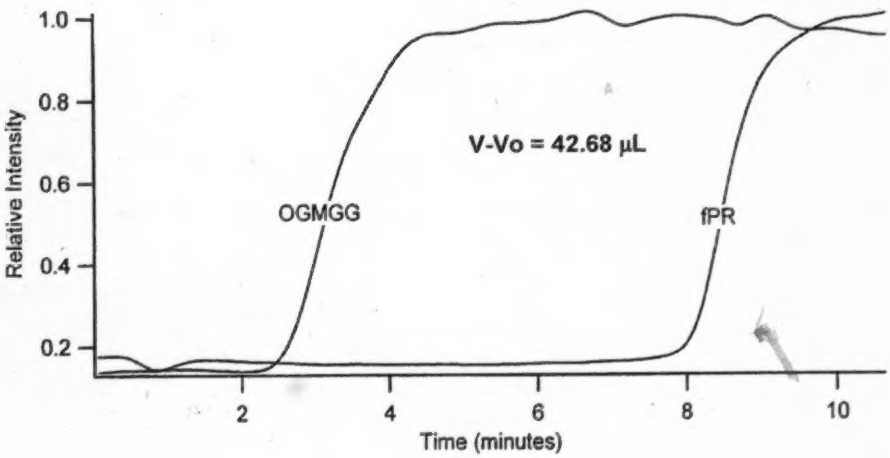

Fig. 2-3. Example of a typical extracted ion chromatograph for the bovine thrombin column. (A) The concentration of OGMGG is 1 μM and fPR is at 5 μM. The elution time (V-Vo) of fPR at this concentration is approximately 5.5 minutes. (B) The concentration of OGMGG is still 1 μM, but fPR is now at 2 μM. The elution time (V-Vo) of fPR at this concentration has increased to approximately 10 minutes. Refer to Table 2-1 for a full analysis of concentration and elution time data. Note the difference in time scales between Figure 2-3 and 2-4.

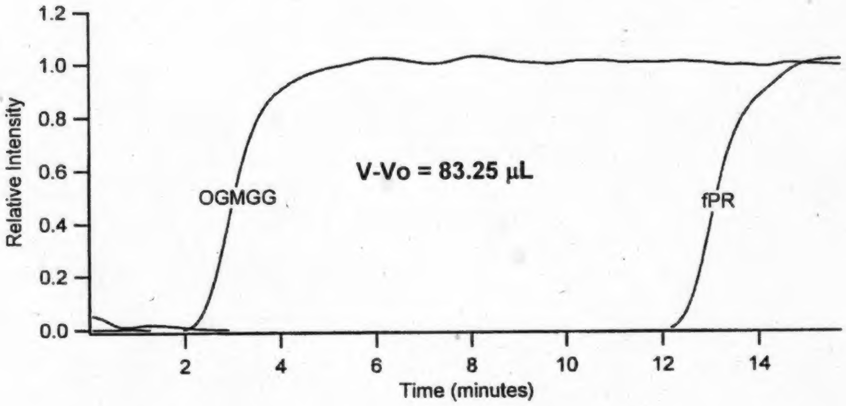

Fig. 2-4. Example of a typical extracted ion chromatograph for the bovine thrombin column with a lower ligand concentration. (A) The concentration of OGMGG is 1 μM and fPR is at 5 μM. The elution time (V-Vo) of fPR at this concentration is approximately 5.5 minutes. (B) The concentration of OGMGG is still 1 μM, but fPR is now at 2 μM. The elution time (V-Vo) of fPR at this concentration has increased to approximately 10 minutes. Note the difference between the time scales between Figure 2-3 and 2-4.

$V\text{-}V_0$	[X] μM	$1/(V\text{-}V_0)[X]$	1/[X]
125.99	1	0.0079371	1
120.55	1	0.0082953	1
120.52	1	0.0082973	1
85.37	2	0.0058568	0.5
89.98	2	0.0055567	0.5
83.25	2	0.0060060	0.5
44.40	5	0.0045045	0.2
45.38	5	0.0044072	0.2
42.68	5	0.0046860	0.2
24.75	10	0.0040404	0.1
25.06	10	0.0039904	0.1
24.19	10	0.0041339	0.1

Table 2-1. Collection and analysis of data to determine the Kd of fPR and the Bt of the bovine thrombin affinity column. Using Equation 1 from frontal affinity chromatography theory (V-Vo) = Bt / (Kd + [Xo]), the data is plotted in Figure 2-4. The analysis is analogous to treatment of data for a Lineweaver-Burke plot in enzyme kinetics.

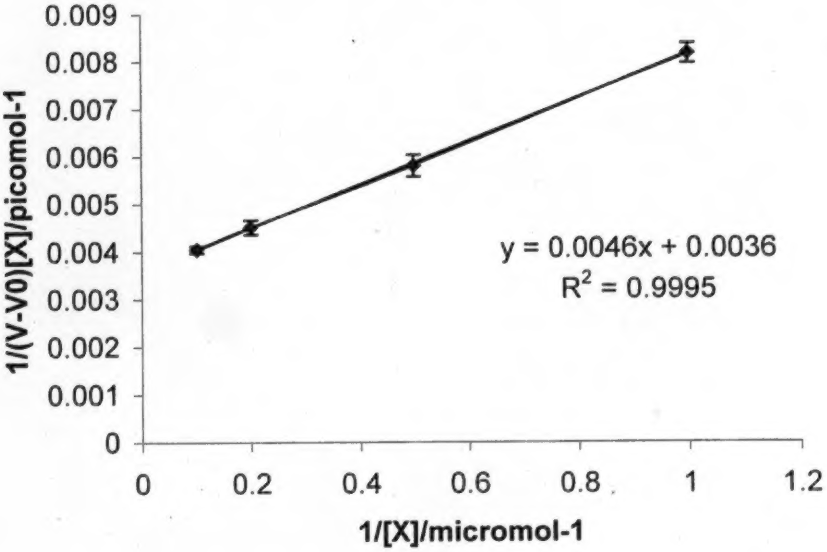

Fig. 2-5. Determination of the column capacity and affinity of fPR to bovine thrombin by FAC/MS. Data points represent the average of three replicates and the error bars represent +/- standard deviation. The R^2 values were calculated using linear regression analysis. The Bt was calculated to be 278 pmol and the Kd was 1.28+/- 0.1 μM.

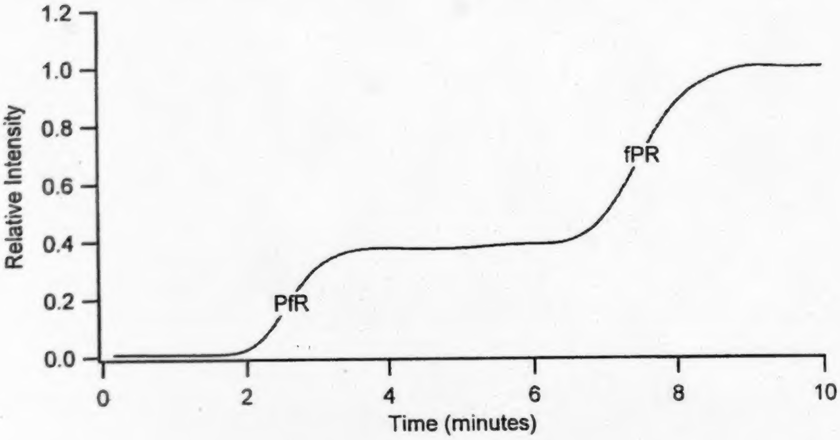

Fig. 2-6. Resolution of fPR and PfR by the bovine thrombin affinity column. The concentration were approximately 5.6 and 2.8 μM respectively. The compounds have the same *m/z* value and thus are both contributing to the breakthrough curve. The "step" pattern is therefore indicative of isomers with different affinities for a receptor.

4. Discussion

FAC/MS is a useful tool for screening mixtures of potential ligands against a variety of targets, including the serine protease thrombin. Previous studies using human thrombin have demonstrated the potential of FAC/MS to select the known ligand fPR out of a background of 99 other peptides (including PfR), however no further analysis was published.[8] These experiments demonstrate that FAC/MS is capable of quantifying the binding affinity of a known serine protease inhibitor to its target, and thus may be applicable to inhibitors of other serine proteases as well.

Preparation of the receptor for immobilization is the most labour intensive task in setting up a FAC/MS assay. Protection of the active site is sometimes required, particularly if it contains reactive groups which can react with the biotinylating agent. Substantial reductions in activity (>70%) have been reported even though care has been taken to protect the active site by inclusion of a known ligand during biotinylation.[12] There are two points to consider to obviate this potential problem. First, because of the inherent sensitivity of the mass spectrometer and the kinetics of receptor-ligand interactions, very little of the receptor is required for the preparation of the column. Second, unlike the majority of enzymatic assays, which often cannot recycle the enzyme for additional assays, the column may simply be washed and reused for multiple rounds of screening. Indeed, one column was used for over 300 runs over a sixmonth period and still retained 95% of its initial activity.[11] Other means of biotinylating proteins have been reported such as site-specific post-translational biotinylation of recombinant proteins in *E. coli*, which would simplify the preparation of the receptor (enzyme) for immobilization and likely preserve acitivity as opposed to the random chemical biotinylation of purified proteins.[4]

The Kd for fPR for immobilized thrombin was found to be approximately 1.28 +/- 0.1 μM by FAC/MS analysis. The Ki for thrombin was found to be approximately 8 μM.[3] The two measurements differ by approximately six fold. It should be noted however that a Kd measured by FAC/MS and a Ki measured by solution phase enzyme kinetics are different measurements, and in some cases, have varied as much as thirty fold for individual compounds.[12] This discrepancy occurs for a variety of ligands such as steroid hormones[2], but there has been no satisfactory explanation for these differences yet. However, on average, the differences between Kd and Ki measured are about an order of magnitude.[12]

The conditions under which each Kd and Ki measurements are made may be completely different. For instance, purified proteins are often found in high salt buffers and with detergents or other additives to prevent aggregation and/or precipitation. Many of these buffer components are not necessary to retain activity of the receptor once it is immobilized on the affinity columns because the individual protein molecules are likely spatially separated from one another when bound in the biotin binding site of streptavidin. But some agents such as detergents and salts which can decrease non-specific interactions are not often unsuitable for ESI-MS instruments. Therefore one major limitation of the FAC/MS system may be that not all proteins will be biologically active in the buffers that are compatible with the electrospray ionizer mass spectrometer (ESI-MS). Volatile salts are required, such as NH_4OAc, and the ionic concentration should be quite low (sodium and potassium concentrations are problematic above 5-10 mM) to improve the signal to noise ratio.[1] In the case of thrombin, the protease appears to be fully active in low ionic strength buffers and does not appear to require other buffer components for its activity.

In summary, thrombin is a useful model for developing the FAC/MS assay. Furthermore, many infectious diseases such as HIV and HCV, and even bacteria such as Anthrax, rely on proteases for their replication or pathogenesis; thus FAC/MS may be a valuable tool in the rapid screening of mixtures (eg. peptides) for the discovery of novel protease inhibitors against these targets as well. Indeed a mass spectrometry driven approach has been applied to find inhibitors of the anthrax protease for example.[6]

5. References

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Chapter 3

Rapid Screening of a Combinatorial Library Against the Hepatitis C Virus NS3 Protease by Frontal Affinity Chromatography and On-line Mass Spectrometry

1. Introduction

HCV has been identified as a major worldwide health concern, for which there are currently no effective small molecule antivirals.[5] Owing to the success of protease inhibitors against HIV, the NS3 protease of HCV has been identified as a major target for drug discovery. However, efforts to develop high throughput assays for the NS3 protease have met with several obstacles that limit the applicability of each assay. Radiometric, spectrophotometric and fluorescence-based assays have been reported that are suitable for automation.[2,6,8,17,20] However, there are concerns about each of these approaches. In the case of assays that rely upon the enzymatic cleavage of a substrate, the problem is product inhibition.[2,14] The analyses must therefore be performed under conditions of low substrate conversion, otherwise the inhibitor potencies might be overestimated.[16] This subsequently affects the maximal signal level that can be generated by the assay. For assays relying on an internally quenched fluorogenic signal, there are inner filter effects, which result in a non-linear increases of fluorescence with increasing concentration of fluorochrome.[17]

In order to overcome these problems, a ligand binding assay was proposed. It had been demonstrated that a fluorescence resonance energy transfer-based direct binding assay utilizing a fluorogenic hexapeptide ligand was capable of accurately measuring inhibitor affinities as well as association/dissociation kinetics.[15] However, the low inherent fluorescence signal forced the investigators to utilize an assay that was more sensitive. This subsequently led to the development of an analogous scintillation proximity assay (SPA), using a tritiated decapeptide and the NS3 immobilized on scintillation beads in place of the fluorescent ligand.[15]

Although this approach solved most of the problems commonly associated with NS3 assays, the assay is still only capable of screening discrete compounds.[15] In other words, if there is an inhibitor in a mixture of compounds, the assay is able to detect inhibition occurring in a well, but it cannot identify the most potent inhibitor in the mixture. If a combinatorial approach is employed to generate large numbers of compounds for analysis, all available assays for NS3 activity would require either massive parallel synthesis and/or an extensive deconvolution strategy (see Figure 1-6) to identify specific inhibitors. This in turn limits the true power of a combinatorial approach. It is clearly preferable to make a library of compounds, identify the most active compounds out of the library, and synthesize a few discrete compounds for further analysis.

FAC/MS is a high throughput screening method that essentially is an active site binding assay capable of screening libraries.[13] In this study, NS3 was purified with a hexahistidine tag and immobilized on a column of streptavidin coated beads by cloning the NS3 protease domain with an *E.coli in vivo* biotinylation tag.[9] A sample containing a randomized P1 library based on the NS4A/B cleavage junction (ie. Ac-DEMEEX-OH, where X is the randomized P1 position) was continuously infused through the affinity column, and the peptides eluted from the column in parallel with their affinities for the receptor. This allowed for the rapid screening and identification of a known ligand, Ac-DEMEEC-OH, and a novel ligand. Ac-DEMEED-OH, from the mixture of peptides. The finding that these ligands bound to NS3 was subsequently verified by additional experiments, including a ligand displacement approach and solution phase enzyme kinetics.

2. Materials and Methods

2.1 Cloning and expression of an in vivo biotinylated protease domain (NS3-Bio)

The NS3 protease domain, spanning residues 1027-1207 of the viral polyprotein, was cloned from the cDNA of the Hu77 strain of HCV (a gift from

Dr. Jens Bukh, NIH, Maryland, USA) using the following primers: 5'-GGAATTCGGCGCCCATACCGGCATACG-3' and 5'-CCGCTCGAGGTGGTTCCAAACCATTTTCTGAGCGTCCAGGATGTGGTGCAGGGATCCACCACCACCAGATCTCATGGTTGTCCC-3'. Both primers were commercially synthesized (Invitrogen, Mississauga, ON).

The second primer was unusually long for a cloning primer but allowed for the introduction of the biotinylation tag during the PCR cloning of the protease domain. An MJ Scientific programmable PCR machine using an amplification protocol of: 92^{O}C for 2 minutes, (92^{0}C for 1 minute, 54^{0}C for 1 minute, 72^{0}C for 1.5 minutes) repeated for 30 cycles, and finally 72^{0}C for 5 minutes, was used to amplify the protease domain. The PCR product was ligated directly to the TA cloning vector (Invitrogen). Individual sequence verified clones were then subcloned into the EcoRI and XhoI sites of pET-21b (Novagen, Madison, WI) generating the pET21b-NS3/Bio vector. The DNA sequence was verified prior to protein expression. *E.coli* BL21(DE3) cells (gift from Karl Fischer, University of Alberta, Edmonton, Canada) were freshly transformed with pET21b-NS3/Bio, and an individual colony was inoculated into a starter LB culture medium supplemented with 100 μg/mL ampicillin, and grown overnight at 37^{0}C. The starter culture was diluted 1/1000 with LB with 100 μg/mL ampicillin and 10 mg/L D-biotin. When the cell density reached an OD_{600} between 0.3-0.4, the temperature was reduced to 30^{0}C and induction initiated by the addition of 1mM IPTG (Sigma). Cells were harvested 2 hours post induction and immediately flash frozen at –70^{0}C. The cell pellet was resuspended in 15 volumes of 50 mM HEPES, 0.3 M NaCl, 30% glycerol, 0.1% β-octyl glucoside, and 2mM β-mercaptoethanol (pH 7.6). The resuspended cells were lysed by sonication, and clarified by spinning at 100,000 g for 30 minutes. The soluble fraction was applied to 5.0 mL HiTrap Nickel affinity FPLC column (Pharmacia) equilibriated in the same buffer. The column was washed with 10 volumes of buffer. Protein was eluted using the same buffer supplemented with 0.35 M imidazole. The yield was approximately 1mg/L. The level of protein biotinylation was determined by measuring the protein concentration before and after the capture of NS3/Bio with an excess of immobilized streptavidin (Immunopure AffinityPak Immobilized Streptavidin, Pierce) using a modified Lowry protein assay (RC DC, BioRad, Mississauga, Canada). The purity of the protein preparation was estimated from a 12.5% SDS-PAGE commassie stained gel. Next, the proteins were transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia) for 1 hour at 4^{0}C, at a constant current of 150 mA, in Tris-glycine transfer buffer (39 mM glycine, 48 mM Tris base, pH 8.3; 0.037% SDS, 20% methanol) using a Mini Trans Blot Cell (BioRad). The membrane was rinsed twice with TBST buffer (10mM Tris-HCl, pH 7.5; 150 mM NaCl, 0.05% Tween-20) for five minutes. The blot was blocked with TBST buffer and 1% skim milk powder for 3 hours. The blot was washed three times for 5 minutes with TBST buffer. Streptavidin conjugated to horseradish peroxidase was diluted 1/1000 with TBST with 2% BSA and incubated with the blot for 1 h (Pierce). The immunoblot was washed once with TBST and the signal visualized by 1.4 mM of 3,3'-diaminobenzidine (Sigma) in TBST containing a 1/500 diluted H_2O_2 (30% vol/vol) solution.

2.2 Peptides

The substrate inhibitor mixture (P1 library), Ac-DEMEEX-OH derived from the NS4A/4B cleavage junction (where X = Cys, D-Cys, Phe, Lys, Gly, Asp, Thr, Ser or Ala was chosen for the broad chemical functionality). The perptides were prepared by the Alberta Peptide Institute (API, Edmonton) using Fmoc chemistry. This library was chosen based on previously reported work using this hexapeptide lead compound.[7] The library was generated by mixing equivalent amounts of starting resin charged with each of the eight amino acids. The crude library was purified on a Zorbax 300SB-C8 21.2 x 250mm reverse phase HPLC column. Pure single peptides Ac-DEMEEC-OH, Ac-DEMEED-OH and fPR, were also synthesized by API. A fully optimized substrate inhibitor (Ac-Asp-D-Gla-Leu-Ile-[β-cyclohexyl-Ala]-Cys-OH) was obtained from Bachem (Torrance, CA, USA). The NS4A cofactor peptide, pep4AK (KKGSVVIVGRIVLSGK), and a chromogenic substrate for NS3, 5A-pNA, were purchased from Anaspec (San Jose, CA, USA)

2.3 Preparation of Microscale NS3 Affinity Columns

An NS3 affinity column for screening the inhibitor library was prepared by packing streptavidin coated beads (see Chapter 2 m) into PEEK tubing (internal diameter 0.50 mm; length 110 mm; column volume 21.6 μL, Fisher Scientific) and subsequently washing the column with freshly purified NS3 protein solution (500 μL, 0.5 mg/mL) at 4 μL/minute. A second identical blank column (no NS3) was similarly prepared. A third affinity column for single peptide analysis and assay verification was similarly prepared (internal diameter, 0.5mm, length 70mm, column volume 14.7 uL). Free biotin-binding sites in all columns were blocked by infusion of D-biotin (1mL, 0.2 mg/mL in PBS).

2.4 FAC/MS Assay

The FAC/MS apparatus was set up as previously described in Chapter 2. The three syringes (each 1mL volume) contained wash buffer (10mM NH_4OAc, pH 7.5; 0.1% β-octyl glucoside; 5% glycerol, 1mM DTT), analyte solution (wash buffer with sample) or makeup solution (100% acetonitrile). The sample syringes and the makeup syringe were infused simultaneously at a flow rate of 8 μL/min. The column effluent was combined with the makeup flow in a mixing tee to give a total flow rate of 16 μL/min entering the mass spectrometer. For characterization of the eluent, the spectrometer scanned from *m/z* 100 to 1500 in 1.5s in the negative-ion mode. For screening the P1 library, the spectrometer was operated in single-ion-mode (locked on the *m/z* values of the individual ligands) and negative ion mode $(M\text{-}H^+)^-$. A chamber voltage of -3500 V with a grounded electrospray needle, N_2 drying gas flow rate of 4 L/min, and N_2 nebulizer pressure was maintained at 480 mbarr. After each run the column was reequilibriated with buffer. Breakthrough volumes were measured as midpoints in the extracted ion chromatograms. All data were processed with Microsoft Excel software, and figures are presented as IGOR program files.

2.5 Characterization of NS3-Bio Affinity Columns and Evaluation of Kds

The binding constant (Kd) of the Ac-DEMEEC-OH peptide and the column capacity (Bt) of the analytical column (11 cm) were determined experimentally based on Equation 1, which governs the relationship between the retention volume (V_x-V_0), the dissociation constant (Kd), the concentration of a ligand $[X]_0$ and the column capacity (Bt), using the known inhibitor Ac-DEMEEC-OH.

$$(1) \quad (V_x - V_0) = B_t / \{[X]_0 + Kd\}$$

The column Bt of the shorter column (7 cm) used to verify the assay was determined by substituting the known concentration of the Ac-DEMEEC-OH peptide and its retention time into equation 1.

2.6 Solution Phase Enzyme Kinetics

Enzyme assays were performed in a 200 μL reaction at 37^0C in a buffer containing 2.8 μM enzyme, 50mM Tris-HCl (pH 7.5), 30 mM DTT, 0.05% Triton X-100, 40% glycerol and the appropriate amount of 5A-pNA substrate with or without the Ac-DEMEEC-OH and Ac-DEMEED-OH peptides. The hydrolysis of the 5A-pNA substrate was monitored on a 96 well Uvmax microtitre plate reader (Molecular Devices, Sunnyvale, CA) with kinetic capability at 405 nM. The Ki of inhibitors were determined by Line-Weaver Burke analysis using KaleidaGraph software program (Synergy Software, Reading, PA, USA).

3. Results

3.1 Enzyme purification and characterization

PCR primer mutagenesis during cloning introduced a short peptide sequence (LHHILDAQKMVWNH) that is efficiently biotinylated by the E.coli biotin holoenzyme synthetase in vivo during recombinant protein expression.[12] The desired protein was purified to >90% purity by a single Nickel column (Figure 3-1A). There was no evidence of the BirA protein co-purifying with the protease domain as reported in a recent study employing a similar biotinylation tag.[14] Western analysis with streptavidin-HRP confirmed that elution product, NS3-Bio, was also the only biotinylated protein present post-purification (Figure 3-1B). The only other known biotinylated protein in E.coli is the 22 KDa biotin carboxy carrier protein, which is removed by the Nickel purification step.[9] Approximately 50% of the NS3 was biotinylated under the expression conditions used. The enzyme had a k_{cat} of 0.2 and a Km estimated at 1 mM with the 5A-pNA substrate. This corresponds well with the previously reported k_{cat} and Km values of 0.2 and 1mM respectively.[10]

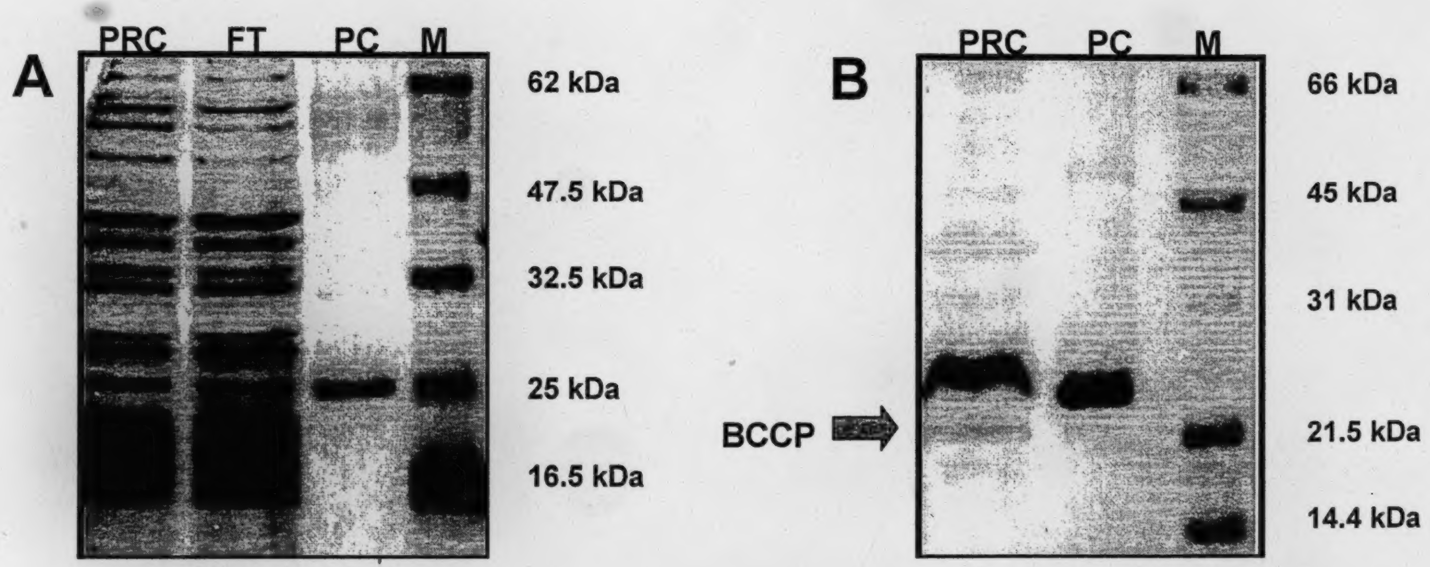
A
PRC
FT
PC
M
62 kDa
47.5 kDa
32.5 kDa
25 kDa
16.5 kDa
B
PRC
PC
M
66 kDa
45 kDa
31 kDa
21.5 kDa
14.4 kDa
BCCP

Fig. 3-1. Purification of biotinylated NS3 protease domain. The expected mass of the NS3-Bio is approximately 24 kDa. (A) Commassie stained 12.5% SDS-acrylamide gel of Nickel column purification of NS3 protease. (B) Streptavidin-HRP Western gel to detect biotinylated proteins. Note the lower band in the precolumn sample at 21 kDa indicated by the arrow. The band likely represents the only other biotinylated protein in *E. coli*, the biotin carboxy carrier protein (BCCP). The abbreviations are: PRC = pre-column purification; PC = post-column purification, FT = column flow through; and M = molecular weight markers.

3.2 Screening of the P1 combinatorial library

The members of the model P1 library were well-represented (Figure 3-2A) and showed minimal non-specific binding on the blank column (Figure 3-2B). The inhibitor library was then infused through the column and the elution profile of each member was monitored (Figure 3-3). Two elements of this chromatograph should be noted. First, the column was able to resolve the two isomers Ac-DEMEEC-OH and Ac-DEMEEc-OH, as suggested by the characteristic "step" pattern in its elution profile. Second, the shapes of the wavefronts themselves should be compared. The wavefronts from the blank column are characteristically sigmoidal (Figure 3-2B). However the wavefronts of the compounds that elute before Ac-DEMEEC-OH and Ac-DEMEED-OH peptides (ie. K,A,T,S,G and F) on the affinity column have a gentle bump or "roll-up" to their wavefront (Figure 3-3). The order of binding (opposite to elution) was: Cys > Asp > Phe > D-Cys >Thr, Ala, Ser, Gly > Lys. The Bt of this column was found to be 1250 pmol and the Kd of pure Ac-DEMEEC-OH peptide was 5.21+/-0.3 μM (Figure 3-4). The relative library Kds are given in Table 3-1.

The protease activity of the immobilized enzyme was also confirmed by infusion of the chromogenic substrate (5A-pNA at 800 μM and 1 mg/mL pep4AK) in the enzymatic assay buffer, at a low infusion rate (2 μL/min), through the NS3 affinity column or the blank column and monitoring the increase in absorbance of

Fig. 3-2. Screening a model library through the blank control column. Extracted ion chromatographs (EIC) from a frontal chromatograph (EIC, normailzed to the intensity of the signal for Ac-DEMEEK-OH). The library was diluted to a final concentration of 0.015 mg/mL for experiments, although the final concentrations of individual peptides are not accurately known. (A) A plot of signal intensity versus *m/z* values of the model combinatorial library in selected ion mode. (B) Control experiment; EIC of the library flowing through a blank column, with 5 μM fPR peptide as void volume marker.

Ac-DEMEE(X)-OH	Relative Kd (μM)
C	6.6
D	7.1
F	11.2
D-Cys	14.9
T	18.0
A	19.3
S	23.2
G	24.1
K	>200

Table 3-1. Relative Kd from the single run of the Ac-DEMEEX-OH library through the NS3 column (Figure 3-3). Assumes the final concentrations of the peptides in the library are the same (approximately 2 μM each peptide). Using equation 1, the elution times from Figure 3-3, and the Bt, determined from Figure 3-4.

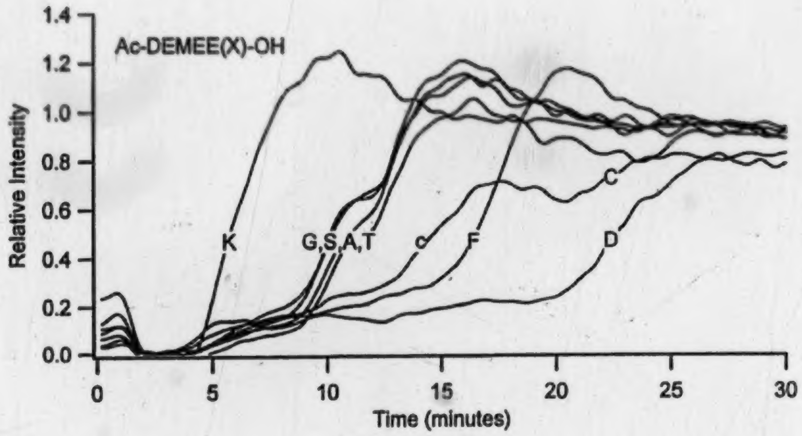

Fig. 3-3. Screening a model library against the NS3 protease affinity column. Extracted ion chromatographs from a frontal chromatograph (EIC, normailzed to the intensity of the signal for Ac-DEMEEK-OH). The library was diluted to a final concentration of 0.015 mg/mL (approximately 2 μM each peptide) for experiments, although the final concentrations of individual peptides are not known. EIC of a single run of the library flowing through the micro-scale HCV NS3 protease affinity column. The void volume marker is omitted as it virtually co-elutes with the Ac-DEMEEK-OH peptide. The relative Kds are given in table 3-1.

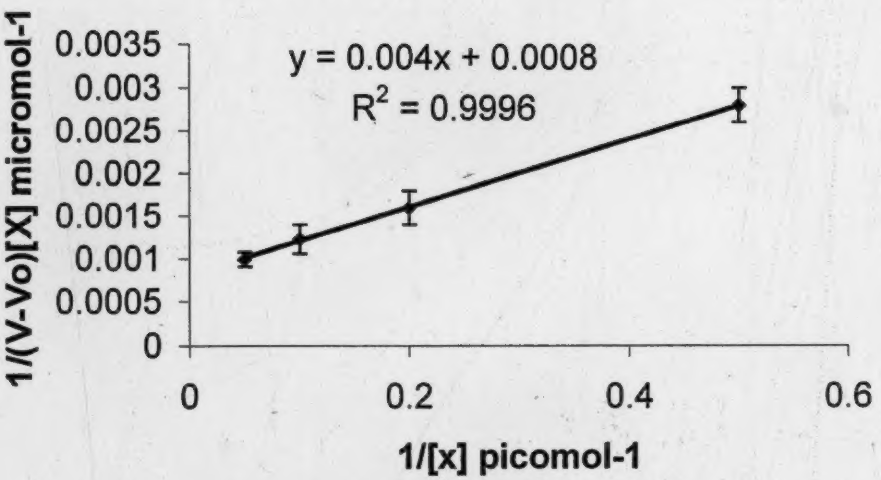

Fig. 3-4. Determination of the column capacity and affinity of Ac-DEMEEC-OH to immobilized NS3 by FAC/MS. Data points represent the average of three replicates and the error bars represent +/- standard deviation. The R^2 values were calculated using linear regression analysis.

the eluant. The absorbance difference (0.295) confirmed the protease was still active following immobilization.

3.3 Confirmation of the FAC/MS assay

The surprisingly late elution time of Ac-DEMEED-OH prompted a closer evaluation, and it was therefore studied as a pure peptide. The order of elution as for the library screening was confirmed using the two most potent individual peptides (Figure 3-5A) and the Kds of single peptides given in Table 3-2. False positives are often a problem in screening assays, but FAC-MS provides a way to rapidly eliminate false positives. In order to prove that the Ac-DEMEED-OH peptide were affects binding at the substrate binding site, the column was first equilibriated with a known potent inhibitor (Ac-Asp-D-Gla-Leu-Ile-[β-cyclohexyl-Ala]-Cys-OH), then the flow switched to a solution containing the Ac-DEMEED-OH peptide and the same inhibitor (Figure 3-5B). The elution time of Ac-DEMEED-OH shifted from approximately 13 minutes (V-Vo = 81.5 μL) in the absence of the inhibitor (Figure 3-5A) to approximately 7.5 minutes (V-Vo = 42.4 μL) in the presence of the inhibitor (Ac-Asp-D-Gla-Leu-Ile-[β-cyclohexyl-Ala]-Cys-OH, Figure 3-5B). This indicates a form of binding competition, although with this data it cannot be determined if it is competitive or uncompetitive.

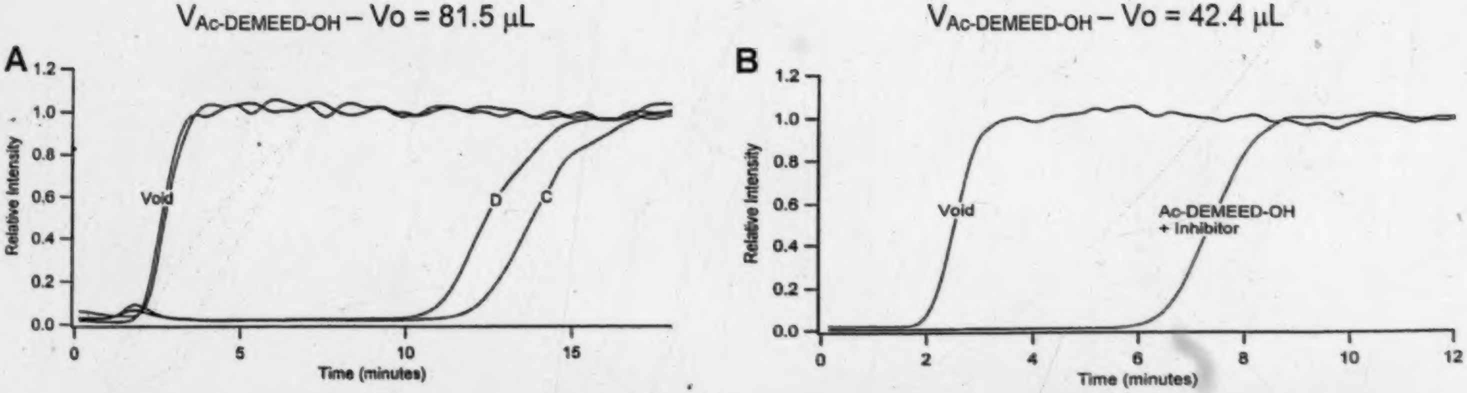

Fig. 3-5. Confirmation of the FAC-MS assay using the 7 cm column. (A) Overlaid chromatographs from individual FAC/MS runs using single 2 μM solutions of the Ac-DEMEED-OH and Ac-DEMEEC-OH peptides through the NS3 column. Void volume marker is 2 μM of the fPR peptide. The order of elution of the two peptides is the same as in the library (Figure 3-3). (B) The elution profile of the Ac-DEEMEED-OH peptide (2 μM) and a potent optimized NS3 inhibitor (Ac-Asp-D-Gla-Leu-Ile-[β-cyclohexyl-Ala]-Cys-OH, 10 μM) in a column equilibriated with 10 μM of the strong inhibitor. The different elution times for the Ac-DEMEED-OH peptide under the two conditions are given above the chromatographs for easy comparison. Note the time scales of the two figures differ slightly.

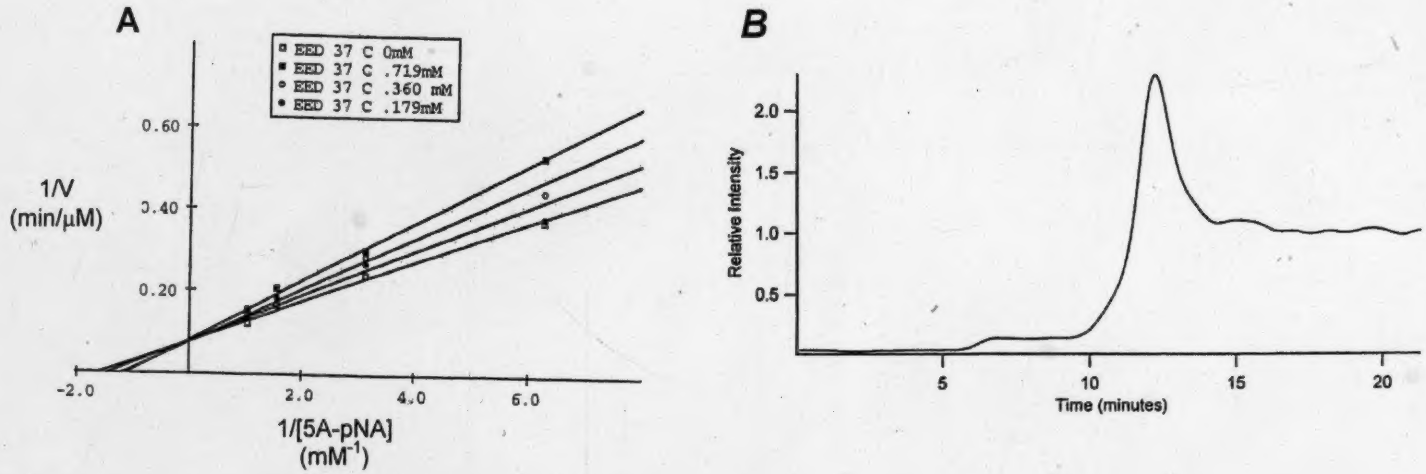

Figure 3-6. Ac-DEMEEC-OH is a competitive inhibitor of the HCV NS3 protease. (A) A Lineweaver-Burke plot of the Ac-DEMEEC-OH peptide using conditions described in the materials and methods. However, the activating peptide, pep4AK was not used in the assay as it formed a precipitate with the other reagents. Single data point analysis. (B) The elution profile of Ac-DEMEED-OH (2 μM) and the strong NS3 inhibitor (Ac-Asp-D-Gla-Leu-Ile-[β-cyclohexyl-Ala]-Cys-OH, 10 μM) infused simultaneously through the 7 cm NS3 affinity column resulting in a "roll-up" effect of the Ac-DEMEED-OH peptide.

Peptide	Ki (mM)	Kd (μM)[a]
Ac-DEMEED-OH	0.975	6.12
Ac-DEMEEC-OH	0.434	5.21

Table 3-2. The inhibition and dissociation constants of peptides Ac-DEMEEC-OH and Ac-DEMEED-OH against the HCV NS3 protease.

[a] The Kd for Ac-DEMEED-OH was determined by a single run using the elution time from Figure 3-5A and Equation 1. The Bt of the column was determined to be 662 pmol.

Cys-OH. In this experiment, a very pronounced "roll-up" effect is observed on the elution profile of the Ac-DEMEED-OH peptide (Figure 3-6). As the Ac-DEMEED-OH peptide is a competitive inhibitor, the "roll-up" effect is the result of competition between it and the stronger inhibitor for the active site. The Ac-DEMEEC-OH is a known competitive inhibitor of the NS3 protease.[14] The Ac-DEMEED-OH peptide however has not been previously studied. The Ki and mechanism of NS3 protease inhibition of the Ac-DEMEED-OH peptide was determined by a Lineweaver-Burke plot was determined to be competitive (Figure 3-6B). The order of elution was mirrored by the order of the measured Kis (Table 3-2).

4. Discussion

The key to FAC/MS is the use of an immobilized receptor to screen ligands based on their relative affinity to the receptor, and the ligand-enzyme binding therefore occurs at a solution/solid interface.[19] The immobilized enzyme may be a good model for membrane bound proteases such as NS3. Biotinylation is perhaps the best method of immobilizing molecules because of its high affinity for streptavidin, and was thus employed as the immobilization strategy. Through genetic engineering, the NS3 protease was efficiently biotinylated (~50%) *in vivo* by the *E.coli* BirA enzyme in this study. The biotinylation likely occurs on the lysine of the (LHHILDAQKMVWNH) sequence appended during cloning.[9] Biotinylation is simplified using this method compared with previous methods where random chemical biotinylation of free lysine(s) was utilized. This technique preserved enzymatic activity as opposed to the chemical biotinylation that resulted in the loss of substantial activity, even though the active site of the enzyme was protected. A similar observation has been reported by another group.[15] For some proteins that lose activity following chemical biotinylation, this *in vivo* biotinylation may be an attractive alternative method of labelling. The enzymatic characteristics of the biotinylated enzyme were similar to previously reported k_{cat} and Km values of the NS3 protease.[10] Therefore, the biotinylation tag did not affect the activity of the enzyme.[15] The Nickel column purification strategy removed the BirA enzyme, which occurred as a contaminant in other preparations using a similar biotinylation tag but not using a hexahistidine tag in their purification approach.[15]

Frontal affinity chromatography allows for the rapid evaluation of a mixture of ligands as potential inhibitors based on their affinity to their target. As a prerequisite, inhibitors must bind to their target, which is also the basis of the SPA assay. Since FAC/MS is capable of monitoring multiple ligands simultaneously, it is a very useful way to screen combinatorial libraries. In the case of FAC/MS an entire library is prepared *en masse*, not as individual peptides, which dramatically decreases the preparation necessary to screen libraries. The order of binding (Figure 3-2C) Cys > Asp > Phe > D-Cys (lower case c) >Thr, Ala, Ser, Gly > Lys demonstrated that the assay was successful at identifying the known ligand (Ac-DEMEEC-OH). Furthermore the assay was able to discriminate the Ac-DEMEEC-OH peptide from its known less potent but isobaric stereoisomer Ac-DEMEEc-OH,[7] giving the characterstic "step" pattern that can be seen (Figure 2-4 and 3-2A). These results also suggested that the immobilized NS3 was biologically active. The finding of Asp and Phe as reasonable ligands was not known prior to the experiment because such substitutions with the NS4A/B junction have not been previously reported. Qualitative analysis of P1 substitutions at NS3-4A *cis* cleavage sites have suggested that Thr should be preferred over Gly, Asp and Phe, as summarized in a recent report, however it would be difficult to predict whether or not its substitution would yield a better ligand prior to running the experiment.[18] Again, the attraction of the combinatorial approach is that a variety of ligands may be screened and subsequently yield some interesting results that may be useful leads for further analysis. As can be seen in Table 3-1, the relative Kds can be estimated (assuming the concentrations are reasonably equal which can be by careful control of the ratio of peptides used[7]) using equation 1. The Kds from libraries are often greater than that of individual peptides likely because of competition for binding for active sites, which essentially reduces the Bt.[19]

The chromatographs themselves are information rich in a FAC/MS experiment.[3] The elution profiles not only report the breakthrough volume, but their shape is also informative. For example, the elution profiles of the P1 library on the blank column are sigmoidal (Figure 3-2B). However, in the NS3 column, the wavefronts display a “bump” or “roll-up” (Figure 3-3). In FAC/MS this phenomena occurs when protein-binding sites near the outlet of the column are occupied by a weak ligand that becomes concentrated relative to the infusion concentration. The weak ligand is then displaced when the front of a stronger ligand appears.[3] The "roll-up" effect occurred with all the ligands that eluted before the Ac-DEMEEC-OH or Ac-DEMEED-OH peptides in the P1 library (Figure 3-2C).

The library screening experiment suggested that the Ac-DEMEED-OH peptide was a potential inhibitor of the NS3 protease, which was surprising. It was only included in the library to show that a variety of molecules with different chemical functionality can be screened by FAC/MS. Its potential as a competitive inhibitor of the NS3 protease was confirmed by solution phase kinetics. It has been shown that the terminal carboxylate is critical for inhibitor activity, and is likely interacting with Lys-136 of NS3 and the oxyanion hole generated by Ser-139 and Gly-137.[1,11,14] One explanation for the binding activity of the Ac-DEMEED-OH peptide is that it may somehow be interacting with this cluster of complementary positive charges. Interestingly, the peptide Ac-DEMEEK-OH, bearing a positive charge at the P1 position, was the first to elute from the P1 library.

The Ac-DEMEED-OH peptide was thought to be a potential competitive inhibitor but was as yet unproven. The equilibriation of the NS3 column with a known potent NS3 protease inhibitor caused a shift in the elution time of the Ac-

DEMEED-OH peptide when the column was equilibriated with it. Since there was a clear shift in the breakthrough volume of the Ac-DEMEED-OH peptide (Figure 3-5 A and B). This suggested that the binding of the Ac-DEMEED-OH peptide was not an artefact or a false positive. Indeed it cannot be a non-competitive inhibitor because its binding was affected by a known substrate inhibitor.

A second experiment with the same more potent peptide inhibitor was able to generate a "roll-up" effect for the Ac-DEMEED-OH peptide when they were infused together into the NS3 column (Figure 3-5B). This is only possible if one ligands affects the binding of the other ligand. It should be noted that this simple feature could be used to rapidly screen even poorly characterized libraries (e.g. natural product extracts) as long as the experimenter has a lead compound to monitor. All the observer must look for is a "roll-up" effect of the lead compound, which would imply that there is a more potent ligand in the mixture than the parent.[3] The most convincing evidence of the nature of the Ac-DEMEED-OH is its Lineweaver-Burke plot, whose common y-axis intersection is characteristic of competitive inhibitors (Figure 3A)

There is a major difference between the Kd (low μM) and Ki (mM) measured in these experiments for actively binding peptides (Table 3-2). The difference varies by over two orders of magnitude. There are several reasons for this difference. First, Kd and Ki are not the same measurement. In previous

FAC/MS experiments, the Kd was often ten-old lower than the corresponding Ki measurement, however in extreme cases it can be thirty-fold less.[19] This is not limited to enzymes either, as it can occur with steroid hormone binding domains and their ligands as well.[4] It is not yet known why these anomalies between the Kd and Ki occur. However in the SPA technique, which also relies on immobilization, the Kd and Ki values are closer to agreement for NS3 protease inhibitors.[15] Second, there are differences in ionic strengths used in the FAC/MS assay and the solution-based enzyme kinetics. The mass spectrometer cannot tolerate non-volatile salt solutions, which can cause problems with the signal-to-noise ratio.[3] It was previously demonstrated that NS3 is susceptible to inhibition by increased ionic strength with a loss of 50% activity with 50 mM NaCl.[16] The effect of NaCl, based on the experimental observation, was that it was increasing the Km of substrates, presumably via destabilization of the P5-P6 interaction. The enzyme is purified with a buffer containing 300 mM NaCl and was diluted twenty-fold for kinetic analysis. Attempts to remove all salt resulted in enzyme precipitation. Therefore the buffer will also contain approximately 15 mM NaCl as a final concentration, which could affect the Ki. One advantage the SPA method has over FAC/MS, however, is its compatibility with salts in the screening buffer. In this case there is a closer correlation between the Kis measured by traditional solution based kinetics and the SPA probe displacement Kis.[15]

FAC/MS has the ability to screen combinatorial libraries efficiently for a variety of targets including viral proteases.. The size of the library was somewhat limited in this experiment, as it was at the limits of detection. However it did present an assay with additional approaches to minimize false positives to screen future combinatorial libraries. With the availability of much more sensitive (up to 3 orders of magnitude) mass spectrometers, the size and complexity of combinatorial libraries that can be screened by FAC/MS is expected to increase.

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Chapter 4

The NS4A Cofactor Peptide and HCV NS3 as a Model of Viral Protein-Protein Interactions

1. Introduction

The ability of proteins to interact with one another is fundamental to biology. Information storage and transfer, cellular structures and enzymatic reactions rely on highly precise recognition events, which often involve the association of two or more proteins.[17] The binding of two proteins may occur with either high or low affinity, but clearly protein-protein interactions do not occur in a random, disorganized way. Viral protein-protein interactions are particularly interesting, as the virus must have evolved specific recognition elements in order to compete with all the other proteins in a cellular environment. That apparent specificity of a protein-protein interaction is an appealing target for antivirals, even though it is generally considered that protein-protein interactions are difficult targets for small molecule intervention.[3,4] However, the recent clinical approval of Trimeris' Enfuvirtide peptide, developed from an earlier observation that gp41 peptides inhibit the fusion event of the HIV envelope proteins with CD4, suggest protein-protein interaction inhibitors may become an important class of antiviral drugs in the future [14].

Enzyme dimerization appears to be another class of interaction that can be disrupted using short peptides derived from one of the associating proteins; this strategy has been used most frequently to disrupt viral enzymes. The HIV protease is an example of an enzyme that can be inhibited with peptides derived from the subunit interface. The protease is functionally active as a homodimer whose interface is an intercalating four-stranded antiparallel β sheet, with the four strands provided by the amino- and carboxy-terminal segments of each monomer.[19,20] The carboxy-terminal sequences are the two central strands. A tetrapeptide derived from the carboxy-terminal sequence was found to inhibit the enzyme with a K_i of 45 μM. Sedimentation analyses were consistent with the mechanism of inhibition being dissociation of the enzyme dimer.[21]

A second example is shown in the studies of the heterodimeric ribonucleotide reductase enzyme from herpes simplex virus. It was discovered that the enzyme could be inhibited by short peptides, in this case derived from the carboxy terminus of the smaller R2 subunit.[5,7] The carboxy-terminal segment serves to anchor the R2 subunit to the larger R1 subunit, by inserting between two helices on the surface of R1.[18] Subsequent extensive analog synthesis produced a much more potent peptidomimetic inhibitor (IC_{50} = 0.3 nM).[10] This type of inhibition is of particular interest in HCV, where the NS3 requires a cofactor peptide, NS4A, for its optimal activity. Furthermore, a minimal binding and activation sequence of NS4A was delineated to the central hydrophobic GSVVIVGRIVLSGK sequence by several research groups and techniques. [2,16]

This same peptide sequence has already been reported to inhibit the NS2/3 *cis* cleavage event, suggesting its potential as a target for antiviral development.[6] Having recently adapted FAC/MS to screen peptides for binding to the NS3 active site, the potential of applying FAC/MS to study the NS3/NS4A cofactor interaction as a model for viral protein-protein interactions was appealing. This may lead to the identification of a peptiomimetic inhibitor of this interaction.

Previous crystallographic data has suggested the NS4A peptide first binds the core of NS3 and then helps direct the folding of the N-terminus of NS3. [11] The interaction of NS4A peptide with the core of NS3 buries 1650 angstroms squared of surface area with an additional 745 angstroms squared from the interaction of with the N-terminal region of the protease, once it has completely folded to its most active form.[8] A 'typical' interface buries ~1600 angstroms squared which is close in area to the key NS3-NS4A contact region suggesting this site as a target of protein-protein interaction inhibitors.[3] The ordering of the active site as a result of binding by NS4A and the interaction of NS4A with the substrate P' residues results in enhanced protease activity. We hypothesized that truncated sequences derived from the central NS4A sequence may bind NS3 but not activate it. Furthermore, structural data suggested that the side chain interactions between the NS4A cofactor and NS3 can be further optimized. Using FAC/MS, a truncated NS4A sequence was identified and subsequently optimized for binding by screening directed combinatorial libraries for each residue in the sequence. Selected optimal sequences were synthesized as pure compounds and tested for their ability to inhibit the NS4A cofactor activation of NS3. One sequence was found that inhibited the interaction of NS4A with NS3, and was not itself an activator of the protease.

2. Materials and Methods

2.1 Peptides

The libraries were synthesized by the Alberta Peptide Institute (Edmonton) using Fmoc chemistry and the molar ratios were adjusted to give roughly equivalent amounts of each library member. Selected peptides were synthesized by Genemed Synthesis (San Francisco, CA).

2.2 *Column Preparation and Instrument Set-up.*

Biotinylated recombinant NS3 enzyme and the affinity micro-columns were prepared as previously described in Chapter 3. Buffers and columns were identical to those previously described, except the peptides and libraries used in this study. The peptides were monitored as the singly charged positive ion ($M+H^+$) by the mass spectrometer, except the pep4AK, which was monitored as its doubly protonated adduct. ($M+2H^+$)/2.

2.3 Measuring relative binding strength of NS4A peptides

The column capacity (Bt) was determined by infusion of several concentrations of of the known ligand, Ac-DEMEEC-OH through the affinity column, and retrofitted to equation 1. In order to determine either the Kd of individual NS4A peptides or the elution order of the NS4A peptide libraries, the elution time on the blank column was subtracted from the elution time on the NS3 column. For screening combinatorial libraries a stock solution of 2.5 mgs/mL of peptides in DMSO for each positional library was prepared. The stock solutions were diluted 1/50 into analyte buffer, except for the Val-23, Ile-29, Val-30 and Leu-31 libraries which were diluted to 1/100 prior to application to the affinity column.

2.4 Enzyme Assays

All enzyme assays were performed under the same conditions using 50 □g/mL NS3, 50% glycerol, 0.05% Triton X-100, 30 mM DTT, 50 mM Tris-HCl (pH 7.5), 300 mM 5A-pNA, the appropriate amount of NS4A derived peptide (in DMSO), with or without pep4AK, such that the concentration of DMSO did not exceed 6%. Separate control reactions verified that this percent of DMSO did not affect enzyme activity. All NS4A and related peptides were incubated with the enzyme for 30 minutes at 37^{0}C prior to the addition of substrate 5A-pNA. The initial rate of hydrolysis of the 5A-pNA substrate was monitored on a 96 well Uvmax microtitre plate reader (Molecular Devices, Sunnyvale, CA) with kinetic capability at 405 nM. Kinetic analysis was performed with Vmax software. All rates were determined by subtracting the background rate of NS3 (in the absence of pep4AK) from the measured rate of the NS4A activated NS3. All experiments unless noted were done in triplicate. The dose response curve of (NH_2-R-Cha-D-val-R-Cha-I-Cha-NH_2) against NS4A activated NS3 was plotted with Origin software (Northampton, MA, USA) using the following relationship of the initial reaction rates:

Equation 2:

$$\text{\% inhibibtion of NS4A activated NS3} = \frac{\text{rate (NS3+pep4AK+pep3)} - \text{rate (NS3 alone)}}{\text{rate (NS3+pep4AK)} - \text{rate (NS3 alone)}}$$

Results

3.1 Delineating the minimal NS3 binding sequences of NS4A

First the binding capacity of the NS3 column was determined (Figure 4-1). The NS4A cofactor was found to have a high affinity for immobilized NS3 (Figure 4-2). An equimolar mixture of truncated NS4A peptides was infused through the NS3 affinity column and the results demonstrated that the length of the peptide was related to the elution time. Deleting a few of the terminal hydrophilic amino acids resulted in a significant loss of binding (Figure 4-3). However, all the peptides studied did appear to have a significant amount of non-specific binding on the blank column (Figures 4-1 and 4-4). The Kds of the peptides were estimated from the mixture and summarized in Table 4-3. The truncated peptide

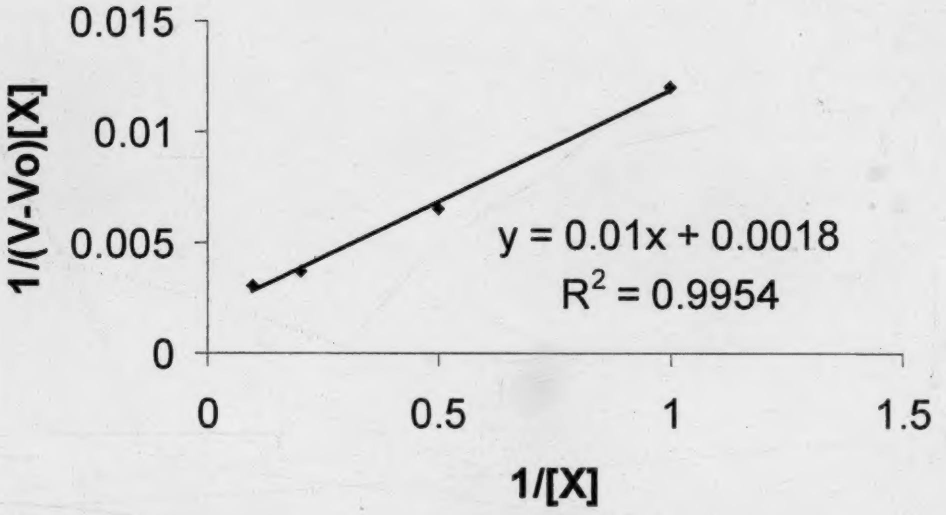

Figure 4-1. Binding capacity of NS3 column for NS4A peptides. The Bt of the NS3 affinity column was rapidly determined with single runs at four different concentrations using equation 1. The Bt determined from the reciprocal of the y-intercept was 556 pmol.

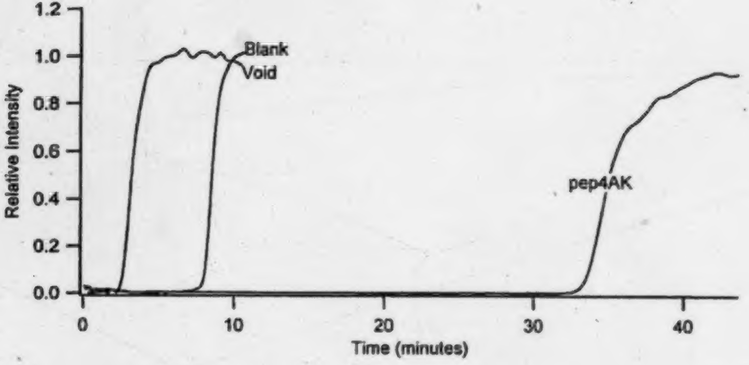

Fig. 4-2. EIC of the NS3 activating peptide pep4AK. The chromatograph of the NS4A derived peptide pep4AK (1 μM, KKGSVVIVGRIVLSGK) overlaid on a blank run using 1 μM of the amino acid D-Val as the void volume marker. Single run.

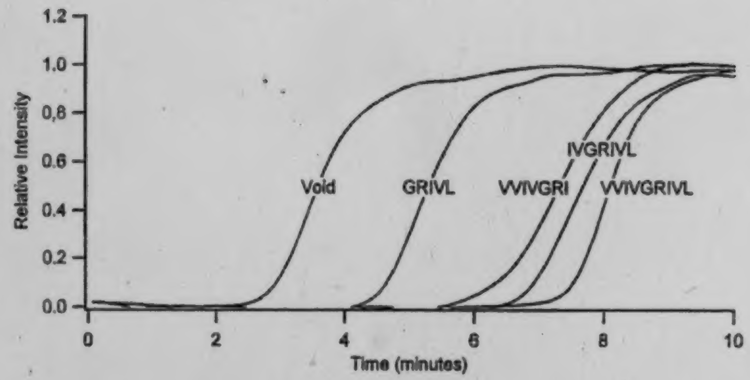

Fig. 4-3. Truncated pep4AK peptides bind immobilized NS3. EIC of single run of truncated peptide sequences of the central hydrophobic region of NS4A, at 1 μM, through the NS3 affinity column.

Peptide	V-Vo (μL)
GRIVL	12.4
VVIVGRI	29.2
IVGRIVL	32.0
VVIVGRIVL	36.4
Pep4AK	253.8

Table 4-1. Elution volumes of pep4AK (Figure 4-2) and truncated pep4AK peptides (Figure 4-3) through the NS3 affinity column.

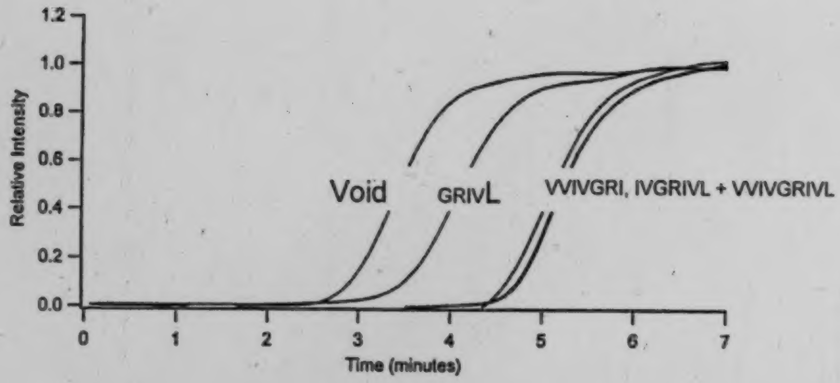

Fig. 4-3. Truncated NS4A peptides bind to the blank column. EIC of single run of truncated peptide sequences of the central hydrophobic region of NS4A, at 1 μM, through the NS3 affinity column. The IVGRIVL and VVIVGRIVL peptides are almost superimposed as they are co-eluting – note the thickness of the last wavefront line.

Peptide	V-Vo (μL)
GRIVL	5.8
VVIVGRI	13.8
IVGRIVL	15.2
VVIVGRIVL	15.2
Pep4AK	24.4

Table 4-2. Elution volumes of pep4AK (Figure 4-2) and truncated pep4AK peptides (Figure 4-4) through the blank column.

Peptide	Kd (μM)[1]	Initial rate[2] mmoles/min/mg
GRIVL	83.2	1.7
VVIVGRI	35.1	1.9
IVGRIVL	32.0	1.8
VVIVGRIVL	25.2	1.9
Pep4AK (KKGSVVIVGRIVLSGK)	1.4	37.4

Table 4-3. Analysis of NS3 binding and activation of pep4AK derived peptides.

[1] Determined by subtracting the difference in the elution volumes between the NS3 (Table 4-1) and blank column (Table 4-2) and substituting back into equation 1. The Bt, 556 pmol was determined from Figure 4-1.

[2] The ability of truncated NS4A peptides to activate NS3 were tested at 360 μM (pep4AK at 20 μM) using the enzyme conditions described in the material and methods. NS3 alone had a rate of 3.7 mmoles/min/mg enzyme. Single experiment shows no activation of NS3 by truncated NS4A peptides

sequences: GRIVL, VVIVGRI, IVGRIVL and VVIVGRIVL showed no evidence of NS3 activation, unlike pep4AK (Table 4-3).

3.2 Scanning combinatorial libraries for each position of the peptide VVIVGRIVL

Using the known crystal structure of the NS4A cofactor and NS3, a biased combinatorial library was synthesized for each residue of the parental peptide VVIVGRIVL. Since the structure was known, amino acid derivatives that were thought to improve binding were included in the library. The libraries were ranked on the basis of their specific elution times on the NS3 affinity column. The results are summarized in Table 4-4.

3.3 Inhibition of NS4A activated NS3 by optimized peptides

Based on the results of the combinatorial libraries, peptide sequences were chosen depending on their binding affinity. In the case where two chemically distinct functionalities were found to bind strongly to NS3 (e.g. $I_{25}\Delta$W,R and $I_{29}\Delta$Cha,R), both were included in the subsequent optimized peptides for screening against the NS4A-activated NS3. The identity of the peptides is given in Table 4-3.

Of the twelve peptides tested only three appeared to show evidence of inhibiting the NS4A activated NS3 (Figure 4-5). Upon closer inspection of the assay wells however, those with peptides 1 and 5 were found to have a fine precipitate. Therefore only the peptide 3 (Pep3) was selected for further

NS4A Residue	*Library Ranking*
Val-23	W>F>tert-L>V
Val-24	F>N,L>D>C>V>>Q,E
Ile-25	W>R>F>Y>K>I>N
Val-26	Cha>>FNO>C>F>Y>V>Q>E
Gly-27	v>>a>n>Q>G>E,D
Arg-28	R>K>Q>V,L>E
Ile-29	Cha>R>W>F>Y>I
Val-30	I>>V>D>N,Q,E
Leu-31	Cha>>W>F>Y,L

Table 4-4. Screening biased positional libraries of the VVIVGRIVL peptide by FAC/MS for binding to immobilized NS3. The libraries included non-natural amino acids: tert-Leu is tertiary leucine, Cha is cyclohexyl alanine, FNO is para-nitrophenyl alanine, and the lower case letters refer to D-amino acids. The substitutions are ranked by elution time on NS3 column subtracted by elution time on blank column.

Peptide	Peptide Sequence
1	W(Cha)vR(Cha)I(Cha)-NH_2
2	W(Cha)vRRI(Cha)- NH_2
3	R(Cha)vR(Cha)I(Cha)- NH_2
4	R(Cha)vRRI(Cha)- NH_2
5	FFW(Cha)vR(Cha)- NH_2
6	FFW(Cha)vRR- NH_2
7	FFR(Cha)vR(Cha)- NH_2
8	FFR(Cha)vRR- NH_2
9	FNW(Cha)vR(Cha)- NH_2
10	FNW(Cha)vRR- NH_2
11	FNR(Cha)vR(Cha)- NH_2
12	FNR(Cha)vRR- NH_2

Table 4-5. Identity of optimized peptide sequences based on library screening.

Fig. 4-5. Screening optimized NS4A subsequences for inhibition of NS4A activated NS3. The concentration of pep4AK was 10 μM and of the peptides screened, 200 μM. The data represents the average of three points with the standard deviation. The control reaction is NS3 with 10 μM pep4AK.

analysis. Pep3 was first tested for its ability to activate NS3, in order to discount the possibility of it being a weak activator. The peptide showed no evidence of NS3 activation, suggesting it was not acting as a weak activator (Figure 4-6). Furthermore Pep3 was able to inhibit NS4A activation of NS3 in a dose dependent manner (Figure 4-7).

3.4 Peptide 3 prevents NS4A binding to NS3

The enzyme assays suggested that Pep3 maybe able to compete with pep4AK for binding to NS3. In order to test that possibility, the elution time of pep4AK was monitored on the NS3 column. The same column was subsequently equilibriated with Pep3 and then the flow switched to a solution of pep4AK and Pep3. The elution time is reduced and is greater than that of the background binding to the blank column, suggesting the peptide is preventing the binding of pep4AK with NS3 (Figure 4-8).

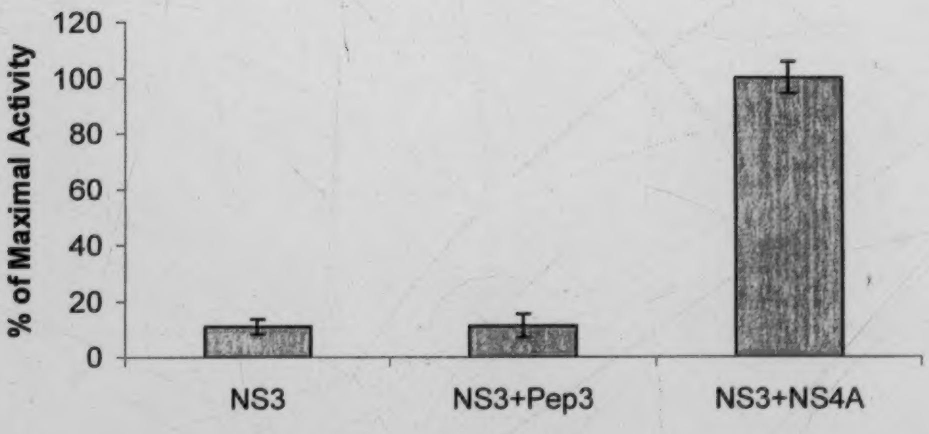

Fig. 4-6. Peptide 3 does not activate the protease activity of NS3. The concentration of Peptide 3 and pepAK was 200 and 20 μM respectively. The graph shows the average of three experiments with standard deviation.

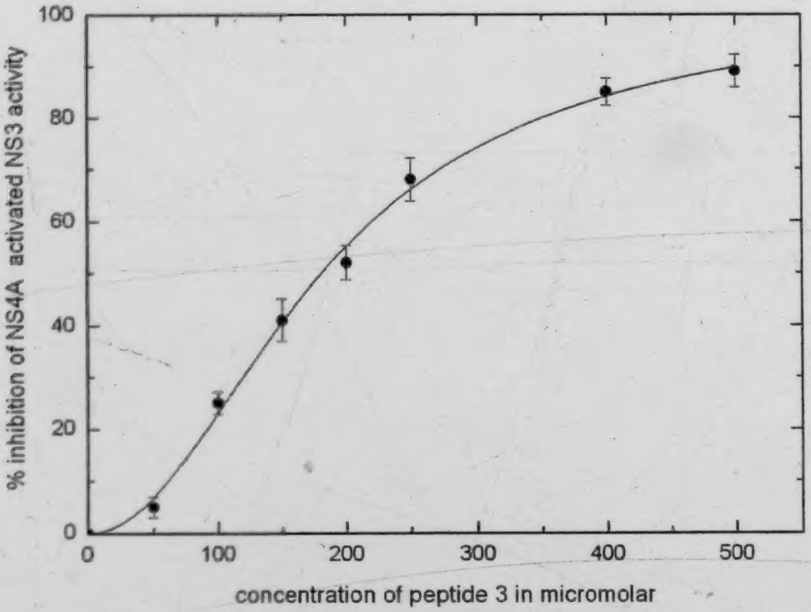

Fig. 4-7. Peptide 3 prevents the activation of NS3 by Pep4AK. The concentration of pep4AK was fixed at 10 μM and concentration of Peptide 3 varied. The percent inhibition of NS4A activated NS3 was determined by equation 2:

$$\%\ \text{inhibibtion of NS4A activated NS3} = \frac{\text{rate (NS3+pep4AK+pep3)} - \text{rate (NS3 alone)}}{\text{rate (NS3+pep4AK)} - \text{rate (NS3 alone)}}$$

The data represents the average of three data points with the standard deviations.

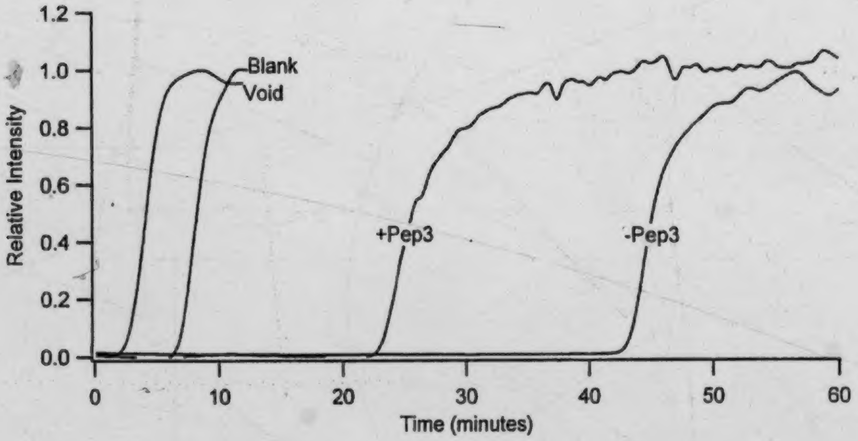

Fig. 4-8. Peptide 3 affects the binding of pep4AK to immobilized NS3. In the absence of Peptide 3, pep4AK (0.6 μM) elutes close to 45 minutes, but if the column is first equilibrated with 25 μM of Peptide 3, the elution time is reduced to approximately 25 minutes. Also shown is the elution time of the void volume marker D-Valine, and pep4AK through a blank column (1 μM each).

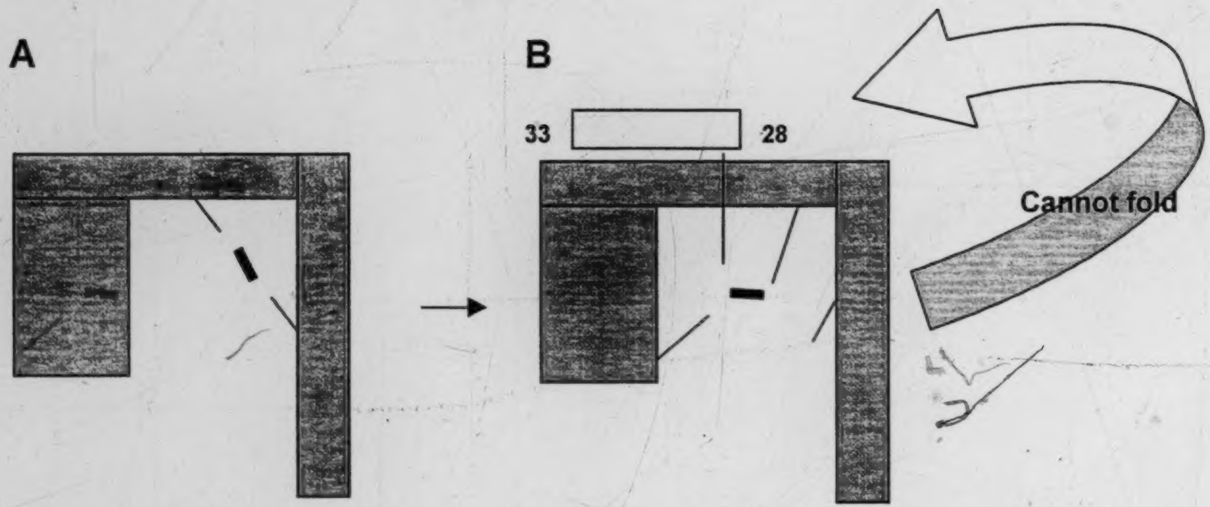

Fig. 4-9. Possible mechanism of Peptide 3 inhibition. (A) There is a key salt bridge (bold line) between Glu-30 (E)and Lys-68 (K) of NS3 which forms a type of "hinge". The N-terminus of NS3 is in an extended conformation. (B) Peptide 3 binds at a hydrophobic site of NS3 similarly as the NS4A residues 28-33 might. The Arg-28 of NS4A, together with Arg-92 of NS3 form a salt bond with Glu-30, which breaks the interaction with Lys-68. However since peptide 3 lacks the N-terminal residues 21-27 of NS4A and therefore the remaining N-terminus of NS3 cannot fold to its final active conformation. Compare to Figure 1-3.

4. Discussion

The association of NS3 and NS4A is one of the best understood protein-protein interactions in HCV biology. Furthermore, as the region of NS4A that binds and activates NS3 (GSVVIVGRIVLSGK) is comparatively small and conserved, it is an excellent model to study its interaction with NS3 by FAC/MS. As evidence of its usefulness, one recent report has utilized reverse-phase HPLC coupled with electrospray ion trap mass spectrometry as a method of determining the stoichiometry of noncovalent complexes using NS4A-NS3 as a model.[13] After calibration, this method is able to determine the stoichiometries of binding, which FAC/MS cannot. The reverse-phase HPLC may be useful in quantifying non-specific binding, however this method is unable to estimate the affinities of binding at present, which FAC/MS can.

The initial binding results were consistent with previously published data suggesting that the minimal sequence containing GSVVIVGRIVLSGK is sufficient for binding and activation. [16] The Kd was determined to be approximately 1.8 μM and the kinetically derived constant was approximately 20 μM for the enzyme derived from the same genotype. [9] The Kd values derived in the case of the pep4AK peptide are not dramatically different from the literature values unlike the substrate based inhibitors (Chapter 3). One explanantion may be that the predominant interactions are hydrophobic, not electrophyllic, and subsequently are less sensitive to the effects of salt. Truncation of the terminal hydrophyllic residues (GS from the N-terminus and GSK from the C-terminus) leaves only the hydrophobic sequence but clearly shows a dramatic loss of binding strength (Table 4-3). The truncated peptides also did not possess any NS3 protease activation activity at concentrations well above their experimentally determined Kds (Table 4-3). The crystal structure revealed that all the main-chain carbonyl and amide groups of NS4A, except V23 and L31, form hydrogen bonds with NS3. Therefore, although the same terminal residues may not contribute significantly to hydrophobic interactions or have much buried surface area, they clearly are important for overall binding strength. Indeed, they can be replaced with other residues such as alanine, serine or aspartate with little effect on the activity of NS4A to activate NS3. [15] One important point is that the significant involvement of the peptide backbone with binding would likely complicate the design of inhibitory peptidomimetics, unless some suitable surrogate could be utilized.

The preliminary experiments demonstrated a significant amount of non-specific binding for the NS4A derived hydrophobic peptides (Figure 4-4). The comparatively good ligand, the full length pep4AK, had a significant amount of non-specific binding. The majority of ligands that have been studied to date by FAC/MS have been carbohydrates and their derivatives, which are typically uncharged hydrophilic compounds. The nature of these compounds is well suited for FAC/MS, exhibiting little non-specific binding. It can be clearly seen that there is a significant problem with non-specific binding with hydrophobic peptides in these experiments (Figure 4-2 and 4-4). Other *in vitro* binding assays used to study protein-protein interactions also have problems with non-specific binding and thus usually include either a washing step, often including detergents, such as in phage display or ELISA-type assays. This is a common problem with binding assays in general and thus should be expected in FAC/MS as well. It is unfortunate that certain detergents such as Triton cannot be included to reduce non-specific binding.

The peptide sequence VVIVGRIVL was chosen as the template for attempted optimization of inhibition. In the crystal structure of NS3 and the small NS4A cofactor peptide, these terminal residues are well buried, even in the absence of complete folding of the N-terminus of NS3. That was true of the full-length cofactor peptide pep4AK however, and may not have been true for truncated sequences. Another reason for using this sequence is that it is shorter that the full-length sequence, therefore it would make the synthesis more economical. By the same reasoning, once optimal sequences were determined, the peptide was further reduced by two residues, in order to decrease the total number of peptides necessary to screen all the combinations of optimal sequences. The eventual intention was to use these peptides in an HCV cell culture model, and longer peptide sequences are known to enter cells less efficiently than shorter peptides.

In retrospect, it would have been better to use the full-length pep4AK as a template for scanning mutagenesis. In this case the non-specific binding would have been reduced to a smaller fraction of the overall binding, and likely made ranking of the libraries much more straightforward. The extent of non-specific binding was not predictable at the start of the experiments.

Regardless of the problems with non-specific binding, a sequence was found in the FAC/MS screening assay, which appeared to prevent pep4AK activation of NS3. Peptide 3, (NH_2-R-Cha-D-val-R-Cha-I-Cha-NH_2), which includes optimal sequences spanning I_{25} to L_{31} has several interesting features. First, the arginine at R_{28} could not be improved upon by any other substitution. This suggested that this residue is important for NS4A binding activity. This same residue has been found to be critrical for NS4A activation of NS3, and its substitution led to the development of the first competitive inhibitor of NS4A activation of NS3.[15] It also suggested that arginine is required for specific binding to occur, since substitution with other hydrophobic residues such as valine did not confer better binding. Second, it was predicted from the structure that a D-amino acid might be tolerated at G_{27}, which is absolutely conserved in all strains of HCV.[8] In fact, altering this residue, even to the comparatively small alanine residue, results in a loss of activity, which likely explains the lack of a side-chain at this position. In contrast the crystalline structure predicted a D-amino acid residue such as D-Val might be capable of binding at this residue which was confirmed experimentally by FAC/MS. Third, the selection of the bulky hydrophobic Cha residues at the other positions is not surprising as the amino acids in these positions were predominantly hydrophobic. The bulky P4' residue of the substrate (e.g. Tyr) has been shown to be in close proximity to Ile-29 of NS4A,[9] therefore perhaps the bulky Cha residue of Peptide 3 is occupying the site where the Tyr should be.

The conformation of the protease domain with and without NS4A has been determined and may provide a mechanism for Pep3's activity. One possible scenario of NS4A cofactor peptide complex formation with NS3 may be that the residues 28-33 of NS4A first bind NS3. The residue Glu-30 from NS3 flips, due to a favourable interaction with Arg28 of NS4A and Arg92 of the enzyme. This locks the base of the N-terminus into its correct orientation. Perhaps Pep3 is able to mimic this first step of the NS3/4A interaction as it possesses these three key amino acids. However the subsequent step involving the bulk of the N-terminus folding into the required secondary/tertiary structure by interactions with the bound NS4A (requiring residues 21-27) is not possible.[11] Therefore the peptide binds but does not fully activate the protease (see Figure 4-9).

Although NS4A appears to be an attractive target for intervention, it should be noted that, using this model, we employed only the central activating core of NS4A for our study. In reality, full-length NS4A complexes with NS3 in a rapid, intramolecular, co-translation event with subnanomolar affinity *in vivo.* [1,12]

Consequently, it is doubtful the peptides described here would be capable of inhibiting this association in vivo. Nonetheless, the FAC/MS assay did appear to be capable of studying this viral protein-protein interaction, and may be useful in future studies to delineate minimal binding domains and lead to the design of small molecules that may inhibit the NS4A/NS3 interaction.

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Chapter 5

Attempt to Design a Bivalent Inhibitor of NS3 by Linking the NS4A Cofactor Peptide to a Potent Substrate-Based Inhibitor

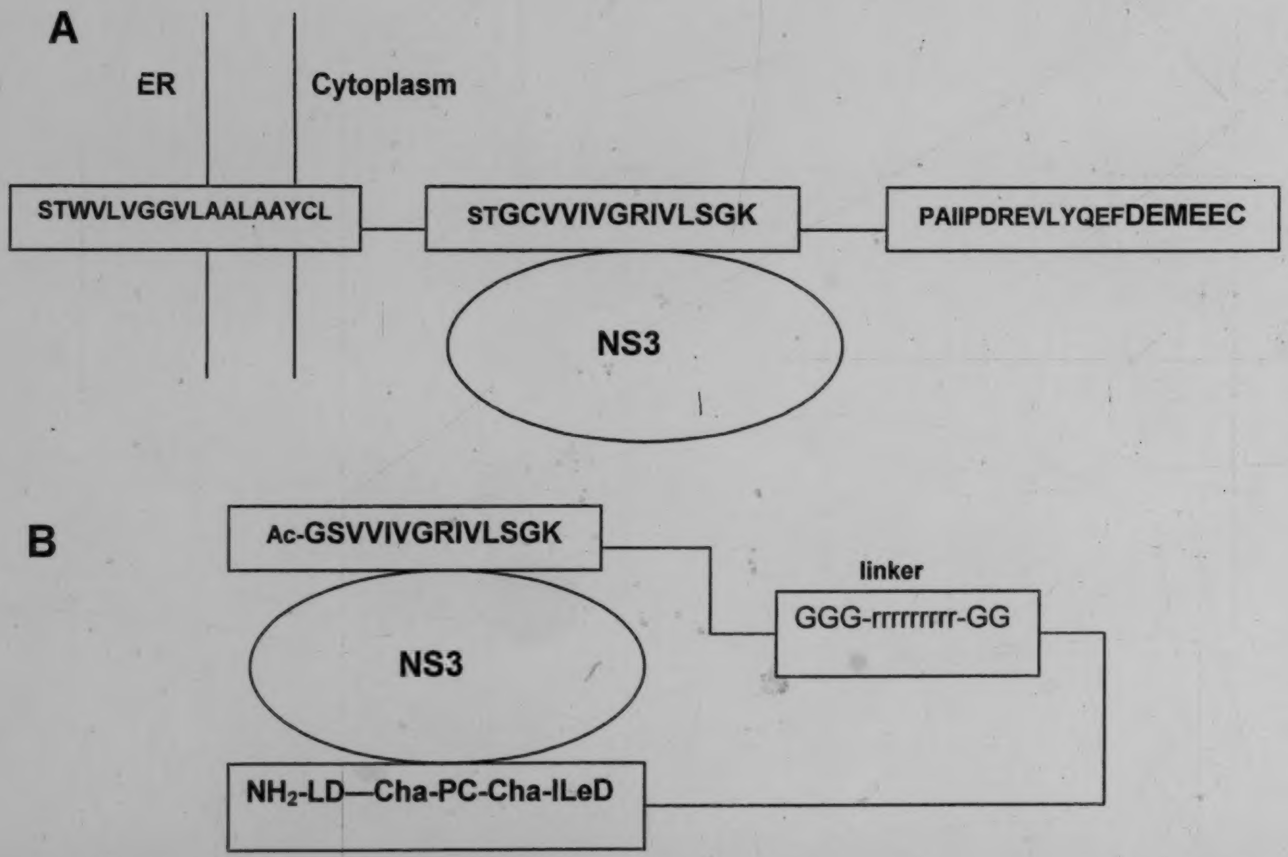
1. Introduction

The interaction of NS3 and its cofactor NS4A is central to HCV replication. Composed of only 54 amino acids, NS4A affects many of the functions of NS3, notably activating the protease by up to a thousand fold, increasing the intracellular half-life of NS3 and localizing NS3 to the ER, where viral replication occurs. [1,4,9] In terms of protease activation, NS4A helps order the active site of the enzyme and increases the affinity of the enzyme for peptide substrates bearing the correct P' residues (particularly decameric peptides spanning P6-P4'). The N-terminus of NS4A (about 20 amino acids) provides a membrane anchor. [2] The central core of the protein is a highly conserved hydrophobic region that is sufficient to bind and fully activate the protease with a K_d of approximately 20 μM. [6] The C-terminal acidic tail of the protein has no known function. [2] It was previously hypothesized, that NS4A can activate its own cleavage in a *cis* reaction from NS4B by the NS3 protease. [10] The NS4A-NS3 binding interaction is known to be subnanmolar *in vivo*, and interestingly is close to the product of the Kd of the central binding and activation domain of NS4A

(GSVVIVGRIVLSGK) and the Ki of the C-terminal cleavage junction (DEMEEC-OH). We therefore proposed that, if a single polypeptide could be synthesized that possessed the central hydrophobic core activating sequence of NS4A linked to a non-cleavable substrate mimic of the NS4A/4B junction it would be an effective inhibitor of NS3. The principle of linking two peptides with two distinct binding sites is not without precedent as a potent inhibitor has been engineered for thrombin by linking substrate based inhibitors with exosite binding ligands. [3]

We elected not to use the Pep3 sequence described in Chapter 4 as part of the bivalent inhibitor for two reasons. First, Pep3 was designed to bind but not fully activate the NS3 protease. This, we predicted, may ultimately decrease the effectiveness of linking this site to the non-cleavable substrate decamer, which was selected for its ability to bind and inhibit NS3 in the presence of the full NS4A activating sequence. Secondly, Pep3 itself may affect substrate binding. Based on molecular modelling, Ile-29 is thought to interact with the P4' of the decameric substrate, helping to decrease the Km of the substrate. Therefore if Pep3 were used, the binding of the linked inhibitors could actually be counter productive as opposed to co-operative, because the bulky Cha group prevents another residue at the P4' position of the substrate from entering the substrate channel.

Previous investigators have described a potent decameric substrate-based inhibitor of the NS3 protease, with a low nanomolar IC_{50} (~ 5 nM) against the J strain.[5] There are 14 amino acids between the end of the central activating NS4A sequence and the P4 residue of the 4A/B junction (Fig. 5-1A). Thus 14 amino acids were felt to be the minimum number of residues required to link the central activating sequence of NS4A and its C-terminal cleavage junction. Since the final peptide was expected to be 38 amino acids in length, the peptide would likely be unable to cross cell membranes. This was not a problem for the large multivalent thrombin based inhibitors as the targets reside on the cell surface. However, the protease activity of HCV resides within infected cells. To circumvent this potential problem, a stretch of nine contiguous D-arginines, were used as the linker together with 5 glycines to comprise the 14 amino acid linker (Figure 5-2B). A short region, termed a protein transduction domain from HIV protein Tat, has been shown to transverse cell membranes in an energy-independent manner, and has been shown to enable even very large (eg. β-galactosidase) proteins to cross membranes, including the crossing of the blood brain barrier.[7] This transduction sequence has been further delineated to require a series of positively charged amino acids, the most potent of which are the guanadino side chains of arginine. Interestingly non-natural amino acids (eg. D-amino acids) with this chemical group retain this transduction ability.[8] The



A
ER
Cytoplasm
STWVLVGGVLAALAAYCL
STGCVVIVGRIVLSGK
PAIIPDREVLYQEFDEMEEC
NS3
B
Ac-GSVVIVGRIVLSGK
linker
GGG-rrrrrrrr-GG
NS3
NH2-LD—Cha-PC-Cha-ILeD

Fig. 5.1. Design of a bivalent NS3 inhibitor. The different functional aspects of the polypeptide are in separate boxes. (A) The sequence and function of endogenous NS4A sequence. There is an N-terminal ER transmembrane anchor, followed by the central NS3 binding and activation sequence in bold, in the box representing the portion of NS4A responsible for this activity. The third box of NS4A sequence is of unknown function, but does posses the N-terminal NS4A/B cleavage junction sequence in bold. (B) The sequence of the bivalent inhibitor. The first box contains the NS4A activation sequence with minor sequence differences, followed by a linker sequence, followed by a non-cleavable optimized substrate inhibitor sequence. The linker is intended to increase cell membrane permeability and mimic the actual length of the peptide sequence. It has been postulated that NS4A can serve as both the cofactor, activating NS3, and substrate simultaneously, hence the idea of the bivalent inhibitor.

linking of peptide sequences of D-arginines and glycines to enhance the ability of a large peptide to cross membranes.

2. Materials and Methods

2.1 Peptides

The chromogenic NS3 substrate 5A-pNA and NS4A cofactor peptide, pep4AK were purchased from Anaspec (San Jose). An optimized hexameric NS3 proteinase inhibitor Ac-Asp-D-Gla-Leu-Ile-[β-cyclohexyl-Ala]-Cys-OH (1) was purchased from Bachem. The NS4A-substrate based bivalent inhibitor Ac-GSVVIVGRIVLSGKGGGrrrrrrrrGGDeLI-Cha-CP-Cha-DL-NH2 (2) was custom synthesized by Multiple Peptide Systems (San Diego, CA).

2.2 Enzyme kinetics

Stock solutions of peptides (1) and (2) were made in water and DMSO respectively and 5 μL used in the reaction. A typical 100 μL enzyme reaction was composed of: 2.5 μM NS3, 50 mM Tris-HCl (pH 7.5), 30 mM DTT, 0.05% Triton X-100, 40% glycerol and 0.639 mM 5A-pNA, and two concentrations of inhibitory peptides. Components were incubated at room temperature for thirty minutes prior to the addition of substrate. Rates were monitored on a Molecular Devices Uvmax spectrophotometer by monitoring the absorbance at 405 nM. Kinetic analysis was performed with Vmax software.

3. Results

The results are indicated as the relative rates of substrate digestion (Figure 5-2). The optimized hexameric inhibitor Ac-Asp-D-Gla-Leu-Ile-[β-cyclohexyl-Ala]-Cys-OH is still the most effective inhibitor of the protease at both concentrations. The Ac-GSVVIVGRIVLSGKGGGrrrrrrrrGGDeLI-Cha-CP-Cha-DL-NH2 peptide was less active despite our prediction that it would be the more potent inhibitor, but it did show a decrease in the relative rate at both the 2.32 and 23.2 μM concentrations when compared to control, suggesting that at least some inhibition was occurring with the bivalent inhibitor.

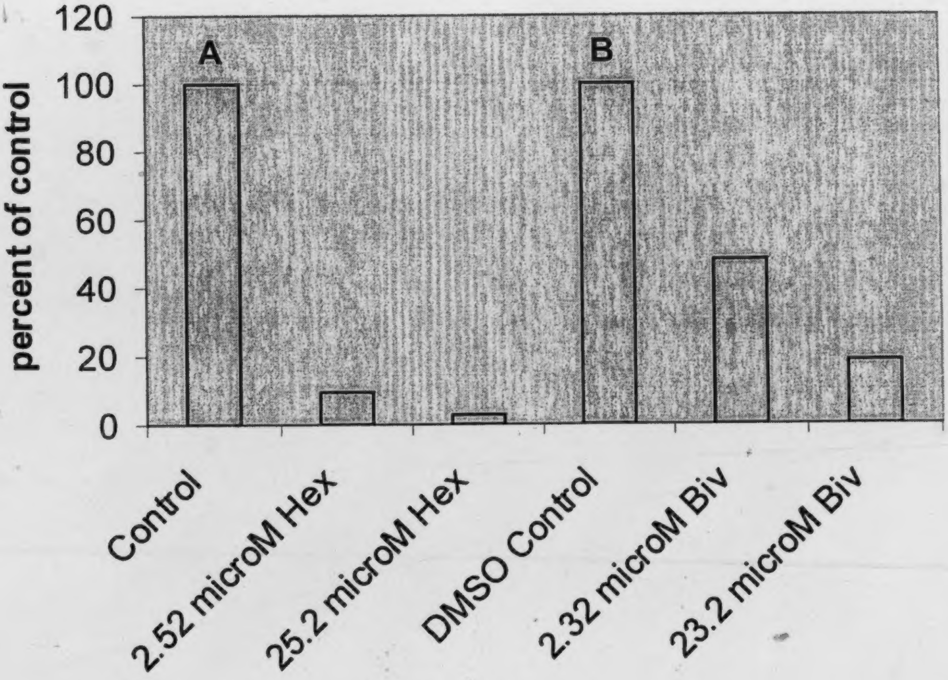


Fig. 5-2. Inhibition of NS4A activated NS3 by a hexameric and bivalent inhibitor. The single experiments represent the relative rate mOD A405/minute of reaction containing inhibitor divided the rate of the control reaction. (A) The reaction rates for the hexameric inhibitor (Hex) are measured relative to the control containing the equivalent volume of water. (B) The reaction rates for the bivalent inhibitor (Biv) are measured relative to the control containing the equivalent volume of DMSO. The DMSO concentration did not exceed 6% and had no effect on the reaction rate as seen in other studies. [6]

4. Discussion

The approach of developing multivalent inhibitors by linking one or more ligands has been used to successfully produce extremely potent inhibitors. In our studies, we attempted to link the NS4A activating peptide with a known potent decameric substrate based inhibitor of NS3, but our results were disappointing. The IC_{50}s against purified enzyme for hexamer and decamer in the J strain were reported to be: 40 and 5 nM respectively.[5] Despite the presence of the more potent decameric sequence in the bivalent inhibitor, it was a substantially poorer inhibitor of NS3 than the monovalent hexameric inhibitor.

There are possible mechanisms that can account for the differences between the two inhibitors. First, the linking of the two sites may impede the binding of one or both to the two binding sites. This may be a function of the chemical nature of the linker, the D-arginine stretch, which is basically oppositely charged to the natural acidic tail of the full length NS4A polypeptide. This problem may be overcome by changing the nature of the linker. However, for an for an inhibitory polypeptide to cross cell membranes, a transduction motif is highly desirable. This motif may alternatively be placed at either termini of the polypeptide sequence, however in these experiments it was placed in the linker to minimize the overall peptide length. Other possible linkers include the native NS4A "linker" sequence, or other highly flexible linkers such as 12-aminododecanoic acid. The latter linker could dramatically decrease the number of amide bonds required in the peptide synthesis to span the two sites. Perhaps generating a combinatorial library in the linker region would allow a rapid assessment of possible inhibitors. Second, the bivalent inhibitor does contain the NS4A activating sequence, and should be able to activate the protease. This increases the total concentration of NS4A in the final reaction mixture, which may be sufficient to keep the protease active despite the presence of the decameric inhibitor.

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Appendix I

Reversion Kinetics of the M512V Mutant of the Duck Hepatitis B Polymerase Conferring Resistance to Lamivudine

1. Introduction

The hepatitis B virus (HBV) is a member of the family *Hepadnaviridae*, which includes the woodchuck hepatitis virus, the duck hepatitis B virus (DHBV) and ground squirrel hepatitis virus.[29,30,44,45] Although it is a DNA virus, it is similar to the retroviruses in that both utilize reverse transcription in their replicative cycles (Figure A-1). After entry into cells, HBV DNA is converted to covalently closed circular DNA (ccc DNA) in the host cell nucleus. Transcription of ccc DNA by the host RNA polymerase generates the pregenomic template which is subsequently transported to the cytoplasm for translation and encapsidation into virus cores. Within the virus core, the pregenomic RNA is copied by reverse transcriptase using a protein primer to initiate the minus strand of DNA synthesis. Using the minus strand of the template, the viral polymerase synthesizes the plus strand of the DNA, resulting in the partially double-stranded viral DNA.[19,36]

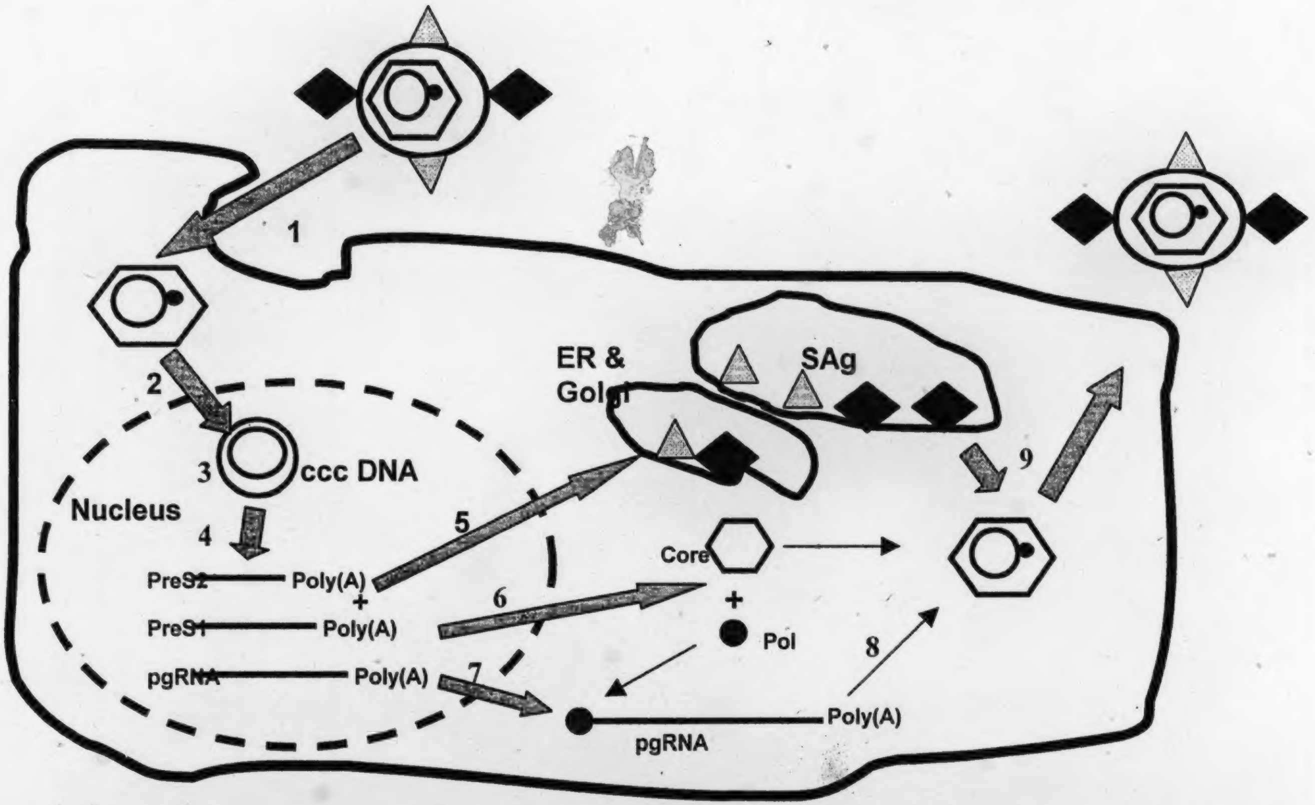

1
2
3
ccc DNA
Nucleus
4
PreS2
Poly(A)
+
PreS1
Poly(A)
pgRNA
Poly(A)
5
6
7
ER & Golgi
SAg
Core
+
Pol
8
pgRNA
Poly(A)
9

Fig. A-1. Generalized life-cycle of DHBV. Steps: (1) attachment and entry, (2) DNA synthesis resumed, core translocates to nucleus, (3) uncoating of genome and viral DNA repaired to form ccc DNA, (4) mRNA synthesis, (5) Surface antigens (Sag) produced, (6) pre-genomic RNA (pgRNA) used to form core and polymerase (Pol), (7) some of the pgRNA associates with Pol, (8) pgRNA+Pol is packaged into a core particle, (9) core particle either recycled to step 2 to amplify the ccc DNA pool or buds through the ER/Golgi membrane, acquiring the envelope and forms an infectious virion.

Unfortunately, over 350 million people worldwide are chronically infected with HBV despite the availability of an effective vaccine. The sequelae of a chronic HBV infection can be cirrhosis and even hepatocellular carcinoma (HCC) [5,31] and approximately 10% of carriers die as a direct consequence of persistent viral infection.[27] Alpha interferon was until recently the only approved therapy available, with only a 30-40% response rate in selected patients.[48] Nucleoside analogues have come to dominate the anti-HBV drug repetoire, the most prevalent of these being Lamivudine.

Lamivudine, the (-) enantiomer of 2'-3'-thiacytidine, is a potent inhibitor of HBV replication.[9,10,16,41] It is phosphorylated by cellular kinases to its active triphosphate form. The nucleotide then competes with cellular dCTP for the viral polymerase and subsequently causes premature chain termination in both the reverse transcription and DNA synthesis steps of HBV DNA synthesis.[43] This antiviral has been used successfully in the long-term treatment of chronically infected patients, who demonstrate histological improvement and significantly reduced viral titres.[4,6,15,20,22,23,37] Furthermore, lamivudine has been beneficial in both the prevention and treatment of reinfection of HBV carriers with liver transplants.[4,7,20,37] Unfortunately, there is often resistance associated with long-term lamivudine monotherapy.

Clinically, resistance to lamivudine monotherapy emerges in two kinetic patterns. In chronically infected patients with high initial viral loads, resistance emerges slowly. The incidence of lamivudine resistance was 24% in chronically infected individuals treated with lamivudine for 1 year in one recent study.[24] In contrast to this pattern, lamivudine resistance in liver transplant patients is as high as 60% at 11 months posttransplant, despite much lower viral loads.[32] The mutations associated with resistance occur most often in the conserved tyrosine-methionine-aspartic acid-aspartic acid motif (YMDD) in the nucleotide-binding site.[3,25,26] A single nucleotide substitution at codon 552 of the reverse transcriptase/polymerase gene (ATG∆GTG, methionine to valine) is one the most prevalent mutations associated with lamivudine resistance.[3,26] Lamivudine resistance in HIV has been shown to result from similar mutations at the YMDD motif.[8,46] Molecular modeling suggests the mechanism of lamivudine resistance of the M∆V mutation is a steric hindrance of the incoming sugar moiety of the nucleoside analogue with the beta branched alkyl group (Cγ2-methyl) of the valine in the nucleotide binding site of the viral polymerase.[13] The "cost" of this lamivudine resistance, however, is decreased fitness in the presence of the drug.[33] This same nucleotide change when introduced at the equivalent position in the DHBV Pol gene also results in resistance to lamivudine.[18]

A key replicative intermediate in a natural hepadnaviral infection is the covalently closed circular DNA (ccc DNA), which serves as the template from which viral mRNAs and the pregenomic RNA are transcribed.[19] Recent *in vivo* studies have shown that the ccc DNA pools have half-lives of several weeks despite continual inhibition of viral DHBV DNA synthesis by L-FMAU or lamivudine and a dideoxyguanosine prodrug, suggesting that the ccc DNA pool is not readily eliminated by antiviral therapy.[1,51] Indeed, viral replication often resumes rapidly when the antiviral therapy is stopped because the ccc DNA pool within the cell has not been eradicated.[35] Thus the status of ccc DNA pools during the course of a hepadnaviral infection are linked to the clinical outcome of treatment and are therefore an important replicative intermediate to monitor.

Previous experiments by Zhang and Summers have shown that there is rapid enrichment of a wild type (WT) virus over a replication-deficient mutant during the initial spread of an infection.[49] Their experiments employed the DHBV mutant, DR1-13, which is partially defective in plus-strand primer translocation due to a single nucleotide substitution. However, their experiments also showed the enrichment of the wild type virus was much less efficient in the chronically infected liver, suggesting the rate of growth of one viral quasispecies over another in a chronic infection decreases over time despite differences in replication rates. Previous research has shown that even though one quasispecies may have a substantial replicative advantage over another, it still must have a minimum threshold during seeding in order to overwhelm the other population[14], a process analogous to clonal interference seen in studies with vesticular stomatitis virus.[34] The dynamic state is possible only if the DR1-13 mutant is able to produce enough progeny to compete with the WT virus and the experiments suggested that once all the available cells are infected there is no more enrighment. To explain these results, the authors proposed a model of “replication space” for DHBV, defined as the potential of the liver to accommodate a replicating virus, which, in the case of hepadnaviruses, is the ability to generate a ccc DNA pool. In their model, one unit of replicative space is able to accommodate only one molecule of ccc DNA. At the cellular level this implies that once a cell has an established ccc DNA pool, it cannot be superinfected by another virus. Superinfection of a persistently infected cell has been reported to be inefficient, lending credence to these assumptions.[38] If this model were correct, then it may explain the long lag period that occurs before resistant mutants of HBV emerge in patients being treated with the nucleoside analog lamivudine.[11,50]

We wished to study the replication space model using the reversion kinetics of the clinically relevant YVDD mutant using the DHBV model. It was hypothesized that if there was an opportunity for a newly revertant virus (ie wild type, YMDD) to establish a revertant ccc DNA pool, such as in an acute infection, then reversion of the serum virus will be rapid. Conversely, if a mutant ccc DNA pool is selected for during an infection, then reversion of the serum viremia should be much slower. This could account for the two distinct kinetic patterns of clinical resistance that arise during lamivudine monotherapy.

2. Materials and methods

2.1 Plasmids for generating extracellular virus (ECV)

The plasimds pCMVDHBV2-MI512VM and pCMVDHBV2-MI512VM-ClaI were obtained from Karl Fischer of the Tyrrell laboratory. Both plasmids have a single nucleotide change in the YMDD motif (AΔG) at position 1703 of the DHBV genome according to the number system of Madart et. al.[28]

2.1. YVDD mutant infectious serum

Serum containing the mutant (**GTG**) was obtained from an animal infected with concentrated mutant virus purified from an LMH cell culture[12] transfected with a plasmid previously shown to produce this infectious YVDD mutant while under constant lamivudine (GlaxoWellcome, Stevenage, UK) selection [20 μg/mL *in vitro*, 40 mg/mL b.i.d. *in vivo*].[16]

2.2. ECV YVDD mutant from transfected cell cultures

LMH cells were cultured in a (1:1) combination of Ham's F12 nutrient medium and minimal essential medium (Invitrogen, Burlington, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM glutamine, 100 μg/mL of streptomycin (Sigma-Aldrich Canada, Oakville, ON), and 50 IU/mL of penicillin G (Sigma) and buffered with sodium bicarbonate. Six-well plates (Falcon) were seeded at $5x10^5$ cells/well and at 18 hours post-plating were transfected with 5 μg of pCMVDHBV2-M512V per plate by the calcium phosphate method.[40] Twenty-four hours post-transfection, the cells were washed with phosphate buffered saline (PBS) and incubated in media containing 40 μg/mL lamivudine. Extracellular virus (ECV) was harvested and purified using a 20% sucrose cushion. Approximately 20-30 mL of culture supernatant was layered over 5-6 mL of a 20% sucrose solution and centrifuged at 24000k for 18 hours at 4^0C. The pellet was resuspended in PBS and used directly for infecting animals.

2.3. Animals

All animals were day-old uninfected Pekin ducks obtained from the University of Alberta farms. For the short-term reversion studies, seven ducks were infected with 100 μL of infectious serum intravenously and their serum collected 3 and 7 days post-infection (p.i.) for genotypic analysis. An additional 12 ducks were infected with 100 μL of ECV from the LMH cultures, and serum collected after 3 and 7 days. At day 7, livers were harvested for ccc DNA analysis.

For the long term studies, 4 animals were infected with serum and treated with lamivudine for 4 weeks at 40 mg/kg b.i.d. Serum was collected and liver biopsies done on all animals. Lamivudine treatment was ceased and the serum viremia collected weekly. One day after mixed populations were detected, animals were sacrificed and their livers harvested.

2.4. Isolation, PCR amplification and sequencing of viremia

Viral DNA was extracted directly from 20 μL of serum by proteinase K digestion, phenol extraction and ethanol precipitation as per published methods.[21] Briefly 20 μL of serum or resuspended ECV was incubated in 80 μL of 50 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM EDTA, 0.1% SDS and 800 μg/mL Proteinase K (Invitrogen) for a minimum of 4 hours. The sample was then extracted with an equal volume of phenol/chloroform (1:1), and extracted twice with an equal volume of chloroform. The DNA was precipitated by adding 1/10th volume of 3M sodium acetate, 10 μg yeast tRNA and 2 volumes of ice cold ethanol, and incubated at –20^{0}C for at least 20 minutes. DNA was pelleted by spinning the tube at 14000 rpm in a benchtop microfuge kept at 4^{0}C for 10 minutes. The resulting DNA pellet was dissolved in 10 μL of sterile water, 2 μL of which used for PCR.

Samples from day 3 required nested PCR using flanking primers 1039 (5'-ctcaagagattcctcagcc-3') and 2771 (5'-gaatctgatttccaata-3') and a second round of PCR with the primers 1137 (5'-accattcctccgtcttcc-3') and 1946 (5'-gtcataccattctcctact-3'). All other serum viral DNA samples collected after day 3 required only a single round of PCR using the standard primers (1137 and 1946) for detection. PCR amplifications were performed in 1xTaq commercial buffer (Invitrogen), supplemented with 2.5 mM $MgCl_2$, 200 μM dNTPs (Invitrogen), 12.5 pmol/ μL of each primer and 1 unit of Taq polymerase (Invitrogen) using 32 cycles of (94^0C/60s, 50^0C/60s, 72^0C/120s). All PCR amplicons were purified using a Qiagen PCR purification kit as per manufacturers instructions (Qiagen, Mississauga, Canada) and sequenced using the primer 1579 (5'-acgggtctactattttagg-3') and the SequiTherm Excel II DNA sequencing kit (Epicentre, Mississauga, Canada) following the manufacturer recommendations. Amplicons from day 3 post-infection were sequenced independently by the University of Alberta DNA core facility using the same sequencing primer.

2.5. Isolation of DNA from duck liver and DHBV ccc DNA specific PCR

The ccc DNA was isolated from sacrificed and biopsied animals was essentially as described.[2] For biopsying, a 16-gauge Jamshidi Menghini soft tissue biospsy needle/syringe (Allegiance Healthcare Co., McGaw Park, IL, USA) was used on 4 week-old animals. Immediately following the biopsy, the tissue sample, approximately 15 mm^3, was washed several times in ice cold PBS to remove most of the contaminating blood. The sample was subsequently homogenized in a 1.5 mL eppendorf tube with a plastic pestle (Kontes, Vineland, NJ) in a 750 μL solution of 5M guanidine thiocyananate, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.3 M β-mercaptoethanol, and 2% Sarkosyl. The mixture was heated at 65^{0}C for 5 minutes to reduce viscosity, cooled to room temperature, and extracted twice with phenol:choloroform (1:1), and twice with chloroform to remove residual phenol. The aqueous layer containing the nucleic acids were precipitated with sodium acetate to a final concentration of 0.3 M with 1mL of isopropanol, at –20^{0}C for 20 minutes. The nucleic acids were pelleted by spinning at 14000 rpm in a microcentrifuge kept at 4^{0}C, and subsequently dissolved in 100 μL of TE buffer containing 5 μg RNase A (Sigma) and 5 μg α-amylase (Sigma). The solution was incubated at 37^{0}C for 1 hour, then extracted with one volume of phenol, then twice one volume of chloroform. The DNA was precipitated as before, washed once with 70% ethanol and briefly air-dried.

A typical PCR reaction to amplify the ccc DNA utilized 25 ng of isolated liver DNA, primers 1454 (5'-ctctccacattacgtag-3') and 2771 at 250 nM, dNTPs at 200 μM each, 2.5 mM $MgCl_2$, 50 μM spermidine-HCl and 2 U of Taq polymerase in the manufacturer's buffer. Cycles for PCR amplification were 94^{0}C for 2 minutes, [94^{0}C for 30 seconds, 53^{0}C for 60 seconds, 72^{0}C for 150 seconds] x 30 cycles. The PCR product was purified using a Qiagen PCR purification kit, and sequenced as described.

3. Results

3.1. *Rapid reversion of the DHBV YVDD (GTG) mutant during an acute infection in the absence of lamivudine treatment*

In order to observe the rate of reversion of the YVDD mutant in an acute infection, seven one-day-old non-congenital Pekin ducks were infected with 100 μL of infectious serum intramuscularly. Their serum was collected 3 and 7 days post-infection (p.i.) for genotypic analysis. The virus population used to infect the animals in this experiment was, as much as possible, of the mutant genotype. The infectious serum was generated by infecting an animal with the ECV from an LMH cell cultures transfected with plasmid pCMVDHBV2-MI512VM. Both the cell cultures and the serum source animal were treated continuously with lamivudine (GSK, Stevenage, UK: 20 μg/mL *in vitro*, 40mg/kg b.i.d. *in vivo*). Evidence of partial reversion of the GTG codon back to ATG in viral DNA populations was detected in 6 of 7 animals at day 3 p.i. (Figure A-2). Complete reversion back to wild type ATG was seen at day 7 in all animals.

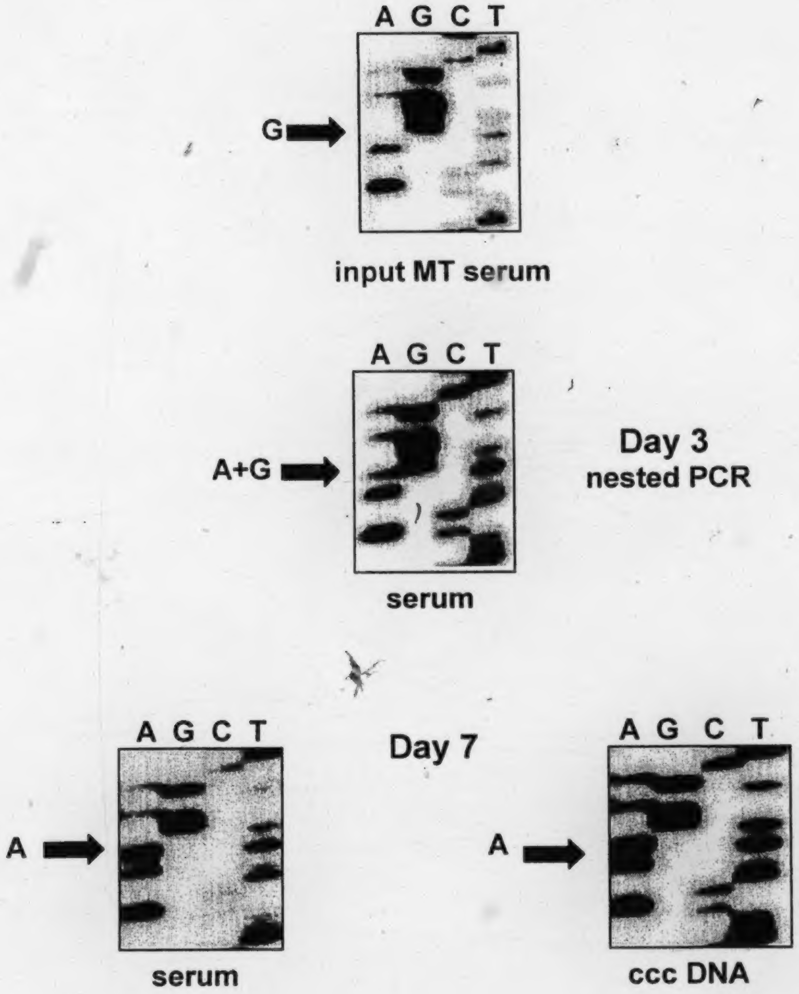

A G C T
G
input MT serum
A G C T
A+G
Day 3
nested PCR
serum
Day 7
A G C T
A
serum
A G C T
A
ccc DNA

Figure A-2. Example of reversion in a non-congenitally infected duck infected with YVDD mutant virus in the absence of lamivudine. The Y(MΔV)DD sequence is: TAT (GΔA)TG GAT GAC. The point mutation is indicated by the arrow. A mixed population is seen in the serum by day 3 post-infection and complete reversion is observed by day 7, in both the serum and ccc DNA populations.

The rapidity of the reversion could have resulted from the presence of wild type virus in the innoculum of serum. To further minimize this possibility, the experiment was repeated using 100 μL of concentrated ECV taken directly from transfected LMH cells cultured in the presence of lamivudine. The ECV mutant genotype was verified by sequencing the inoculum (ie. pooled ECV) prior to infection. Twelve one day-old animals were again infected with 100 μL of ECV concentrate. No PCR product was produced from serum samples collected on day 3 p.i. However, all 12 animals were wild type by day 7 p.i., consistent with the results obtained with the serum inoculated animals.

As the ccc DNA pool is the template for generating new virus in infected cells, the genotype of the hepatic ccc DNA was examined after reversion.[19] After day 7, the livers of the mutant-virus-infected animals were harvested, and the ccc DNA genotype was found to be exclusively wild type (Figure A-2). We were unable to isolate the small amounts of ccc DNA present in livers harvested at day 3 following infection. This experiment suggests that if there is sufficient "space" for a newly reverted virus to infect the liver, as in an acute infection, then reversion can be very rapid and that the revertant virus dominates the ccc DNA pool of most hepatocytes.

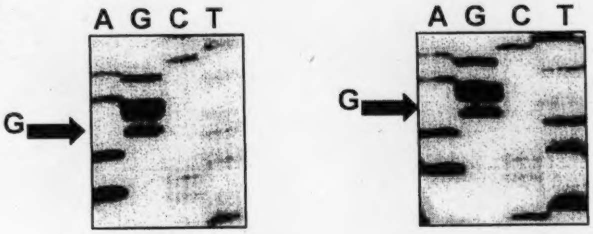
3.2. Reversion is delayed in an established DHBV YVDD infection of lamivudine treated animals following cessation of therapy.

Four one-day-old, non-congenitally infected ducks were infected with the YVDD mutant-containing serum as before and maintained on lamivudine at 40 mg/kg b.i.d. for 4 weeks. At 4 weeks, serum DHBV and ccc DNA obtained from biopsies were amplified and sequenced. Only the mutant was detected in both the serum DNA and the ccc DNA in all animals (Figure A-3). Thereafter, lamivudine treatment was discontinued and animals were monitored weekly for the appearance of wild type virus in their serum.

Following cessation of the lamivudine treatment, wild type virus appeared at week 4 in three animals and at week 5 in the remaining animal. Despite the appearance of wild type virus in the serum, the ccc DNA pool was found to be of the **GTG** or mutant genotype (Figure A-3).

3.3 Reversion is not a result of contamination

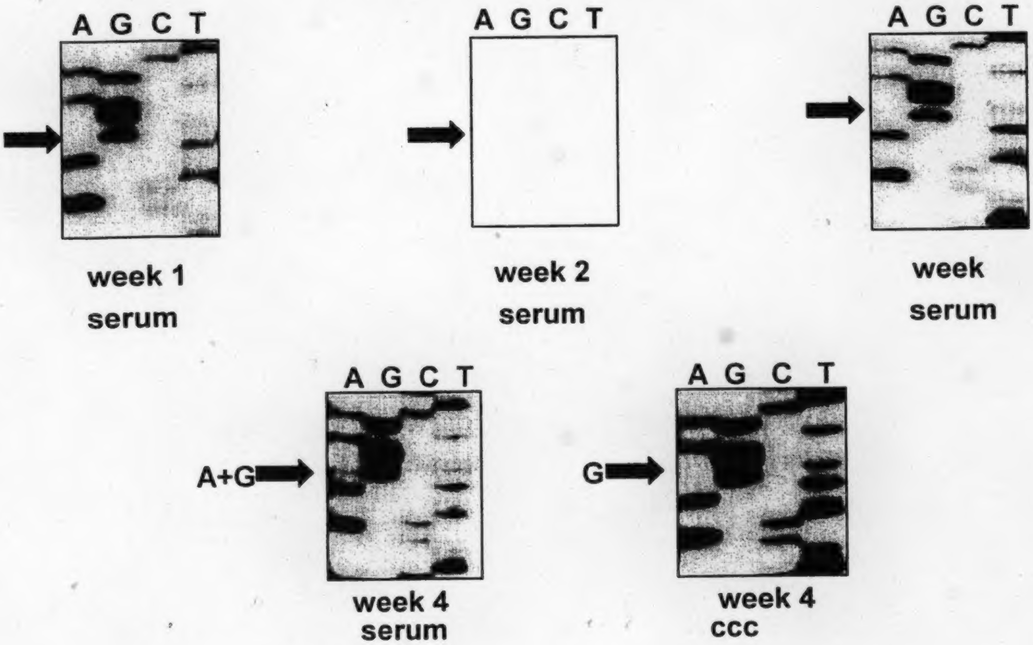
The fact that reversion occurred in all animals *in vivo* in the absence of lamivudine suggested strong selective pressure at the YMDD motif. In order to demonstrate that this was a not a result of contamination of wild type virus from some other unidentified source, a silent mutation resulting in a unique restriction site (Cla I) was co-introduced into the DHBV genome containing the MI512VM



serum ccc

After 4 weeks of lamivudine treatment

Weekly monitoring in the absence of lamivudine



week 1 serum

week 2 serum

week serum

week 4 serum

week 4 ccc

Figure A-3. Example of reversion of a non-congenitally infected duck, infected with YMDD mutant virus, treated with lamivudine for 4 weeks and then released. The point mutation is highlighted. A mixed population in the serum viremia is seen in week four, but only the original input mutant is detected in the liver ccc DNA population.

mutation. Three day-old ducklings were infected with 100 μL of ECV and after 4 weeks, serum viral DNA was harvested, PCR amplified and sequenced (Figure A-4). An aliquot of PCR product was also subjected to Cla I digestion. The results showed reversion at the YMDD motif to the wild type but preservation of the Cla I site, suggesting that there was no outside contamination of wild-type virus from some unexpected source.

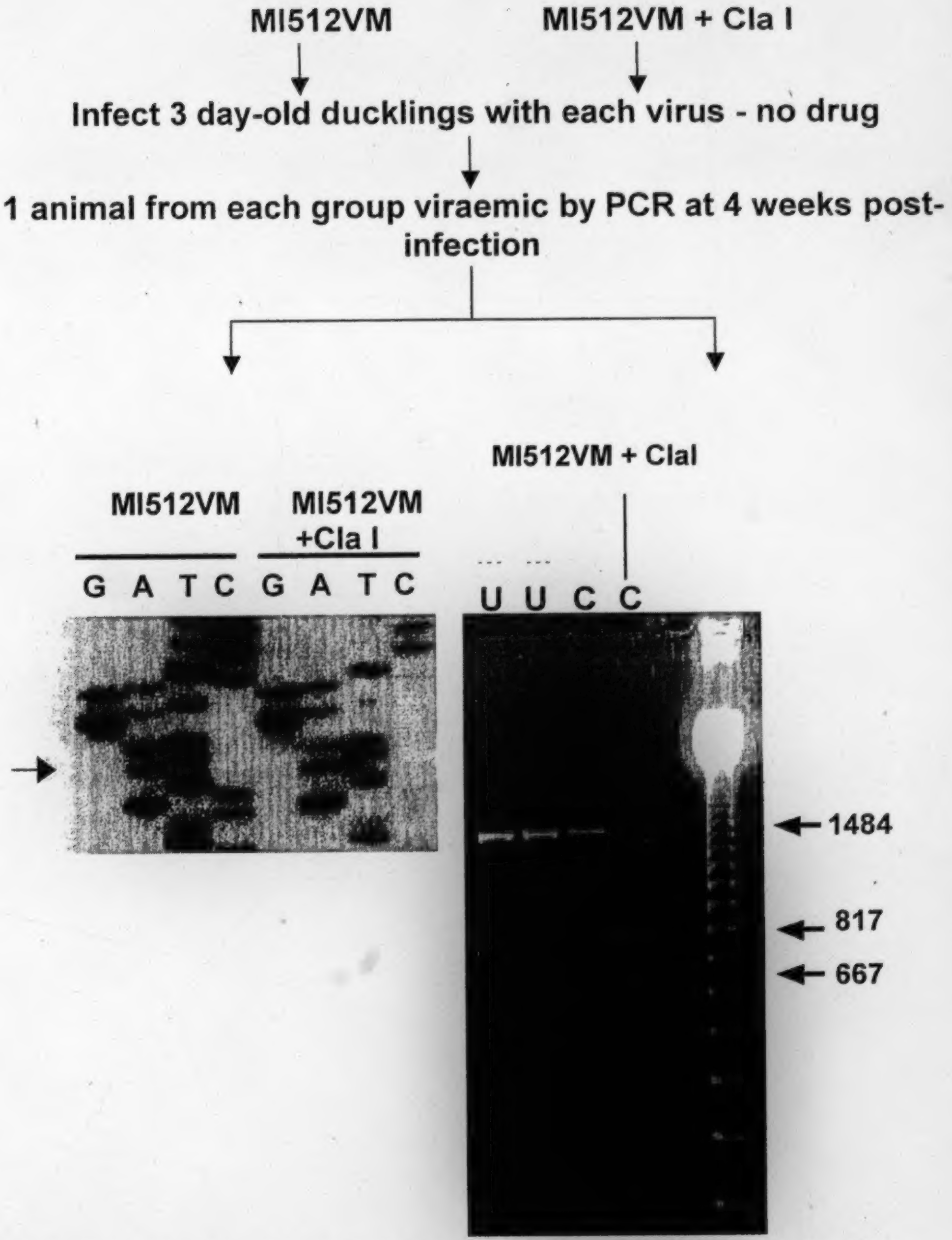

MI512VM
MI512VM + Cla I
Infect 3 day-old ducklings with each virus - no drug
1 animal from each group viraemic by PCR at 4 weeks post-infection
MI512VM + ClaI
MI512VM
MI512VM +Cla I
G A T C G A T C
U U C C
1484
817
667

Figure A-4. Reversion is not the result of contamination from other animals. Two ducks are infected with the MI512VM mutation and the second with the MI512VM mutation and also a unique ClaI marker. Both animals show reversion to wild type sequence as shown by the arrow on the sequencing gel of the serum viremia PCR product. The uncut (U) serum viremia PCR product from both animals migrates at 1484 bp in the ethidium bromide stained agarose gel. However when the serum viremia PCR product of both animals are digested with ClaI (C), the 4th lane, representing the input ClaI engineered mutant, shows the band at 1484 bp is lost, and two faint new bands at 817 and 667 bp respectively appear. . The lane labelled M corresponds to 123 bp DNA ladder.

4. Discussion

The selection of drug resistant mutants on prolonged antiviral therapy remains a major challenge for clinicians. Little is known about the stability of some of these resistance mutations *in vivo*. We therefore investigated one of the most common lamivudine resistant mutants, the YVDD Pol mutation, in the DHBV system. Our results are consistent with the previously proposed "replication space" model of hepadnavirus infection.[49] The rapid reversion observed in acute infection is dependent on the large number of uninfected liver cells present early in the infection. Since DHBV replication has a high mutation rate, in the absence of selective pressure to maintain lamivudine resistance, revertant virus will likely arise before the liver becomes completely infected with drug-resistant virus.[39] Although both populations are observed in serum at day 3 post-infection, wild type DHBV is known to have a substantial replicative advantage over the YVDD DHBV mutant[42] and totally dominates both the serum and ccc DNA populations by day 7. In this scenario there is a large replicative space available for the newly reverted wild type virus to establish a ccc DNA pool in the liver. The rapid reversion of the mutant virus to the wild type virus in naïve animals may also imply that the mutant virus would be unlikely to propogate in a naïve subject, thus making person-to-person spread of the mutant virus very unlikely. Individuals infected with the mutant virus would likely manifest only the wild type virus.

Delayed reversion to wild type in animals chronically infected with the mutant virus is also predicted by the "replicative space" model. In this case, there is little "replicative space" available for a newly revertant wild type virus to occupy and from which to replicate. Other factors should be noted with respect to this model. First, established ccc DNA pools are known to be very stable, even in the face of strict antiviral regimens, which would imply that ccc DNA turnover is not contributing greatly to creation of any new replication space.[1,51] Second, in the older animals that were treated with lamivudine, the liver cell turnover rate is likely to be quite low,[35,49] which would result in few fresh hepatocytes being available for infection. Third, hepatocytes already infected by DHBV exclude superinfection by DHBV virions,[38] even if they have a selective advantage.[47] Therefore in the established infection there is little replication space available for a newly reverted wild type virus to establish a ccc DNA pool, and hence the much delayed appearance of the wild type virus in the serum. Indeed, only the mutant form of ccc DNA was detectable in animals when wild type virus first appeared in the serum. It should be noted that the detection limit of a mixed population of mutant and wild type by sequencing was found to be approximately 20% in similar viral population sequencing experiments .[21] These findings help explain the slow emergence of lamivudine-resistant viruses when patients have a high virus load in the liver, such as with a chronic HBV infection treated with lamivudine, compared to the more rapid emergence of lamivudine-resistance in liver transplant patients on lamivudine when the viral load is low, but the "replication space" is high.

Although the YMDD is a major mutation associated with lamivudine resistance, it is not the sole one nor is it the only site of mutation in lamivudine resistant viruses. Mutations at other sites such as the upstream L512M in HBV in the "B" domain of the HBV polymerase may help increase replication competence of YMDD viruses.[17] Some of these stable mutants are being produced in our laboratory to study their reversion kinetics.

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Summary of Research and Future Directions

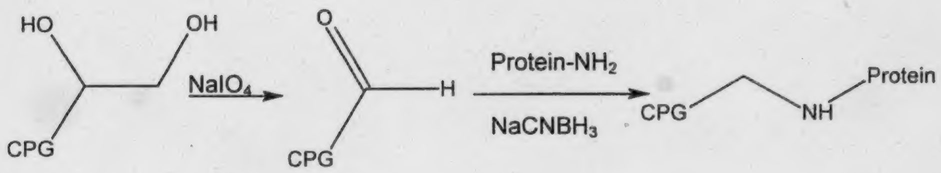
FAC-MS has been previously used to successfully screen combinatorial carbohydrate libraries for both enzyme inhibitors and ligands for lectins. It was of interest to test the capability of the assay for a wider range of medically relevant targets. Early experiments and those in chapter 2 suggested that FAC-MS might be a useful technique for screening libraries of potential protease inhibitors, such as inhibitors of the HCV NS3 protease.

The work with the substrate inhibitor library in Chapter 3 demonstrated that FAC-MS successfully identified the known substrate inhibitor (Ac-DEMEEC-OH) out of randomized library in the P1 position. However the binding affinity (Kd) was found to be substantially lower than enzymatically measured binding constants (i.e. Kis) by Lineweaver-Burke analysis. It is known that electrostatic interactions between the substrate (ligand) and the enzyme (receptor) can be highly sensitive to the absence of salt in buffers, as is often used in ESI-MS compatible solutions. This discrepancy can be explained by the requirement of an acidic residue in the P6 position of an NS3 substrate, where lowering the salt concentration increases the importance of electrostatic interaction. Fortunately, the interpretation of the binding data was not complicated by non-specific binding, as the Ac-DEMEEX-OH library exhibited minimal binding to the blank column. Therefore, as has been the case with mixtures of carbohydrate-based ligands, the system is useful to screen highly hydrophobic, negatively charged molecules provided the "hits" are verified in secondary assays. No comment can be made about molecules that are highly positively charged with this data. However, as the majority of the NS3 protease inhibitor development involved departing from the highly charged N-terminally derived peptide substrate inhibitors towards more hydrophobic molecules and thus the utility of the assay to monitor the binding of hydrophobic molecules became important and the subject of Chapter 4.

The experiments monitoring the NS3-NS4A cofactor peptide interaction in Chapter 4 demonstrated that this well-studied interaction appeared to be less sensitive to the absence of salts in the buffers and was within an order of magnitude of published Kd values. However the non-specific binding to the blank column was substantial. As the majority of molecules that exhibit "drug-like" properties are hydrophobic in nature, this could become a concern if the assay is used to screen other libraries that are hydrophobic in nature. At present, in order to calculate a Kd constant for the truncated NS4A peptides, the specific binding was subtracted by a large non-specific binding component yielding an admittedly crude measurement. It would be far more preferable to eliminate the majority of non-specific binding at the onset of future studies. Unfortunately, the traditional ways to minimize non-specific binding such as addition of detergents (eg. Triton or Tween) can cause problems with signal detection and blocking of the nebulizer.

There are two obvious sources that might contribute to the non-specific binding seen in the experiments of Chapter 4, namely the PEEK tubing and the strepavidin on the coated beads. First the PEEK tubing is comprised of a polymer of poly ether-ether ketone (plastic). The tube is intended to replace stainless steel because of its chemical resistance, mechanical strength and apparent biocompatibility according to the manufacturer. In the case of the hydrophilic molecules, such as carbohydrates and charged peptides, this might be true, however it may be that the tubing is a significant contributor to the non-specific binding. Therefore, reverting back to stainless steel tubing or use of glass capillaries may be preferable.

Second the nature of the column matrix might be changed. Although the *in vivo* biotinylation tag is useful in both preserving enzymatic activity and is a defined site-specfic modification, the fact that it requires streptavidin for its ultimate immobilization might also be a disadvantage of the current system. This is particularly true if the streptavidin itself can provide non-specific site(s) for ligands to bind. One possible way to overcome the use of streptavidin is to use "Glyceryl" coated glass beads (CPG). Briefly, a geminal diol on the short aliphatic extension arm can be activated with sodium periodide to produce a reactive aldehyde group. Then a protein bearing an amino group (eg. lysine) can be coupled to the bead with sodium cyanoborohydride through a reaction referred to as reductive amination:



Unreacted aldehyde might be reacted using an excess of Tris as was analogously done in Chapter 2 to terminate the biotinylation reaction. The advantage of this non-ionic, hydrophyllic coating should be its compatibility with aqueous solutions and theoretically minimal non-specific interactions with hydrophobic compounds. The disadvantage of this system is the possibility of reaction with an important amino group using this random coupling chemistry. Alternatively, other coupling chemistries and matrices could be explored to immobilize the receptor.

Regardless of these issues, the experiments supported the rapid screening of a library by FAC-MS, the strongest binding ligands of which subsequently synthesized in pure form and tested in secondary assays is a legitimate approach to discovering new lead antiviral compounds.